

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION  
International Bureau



BE

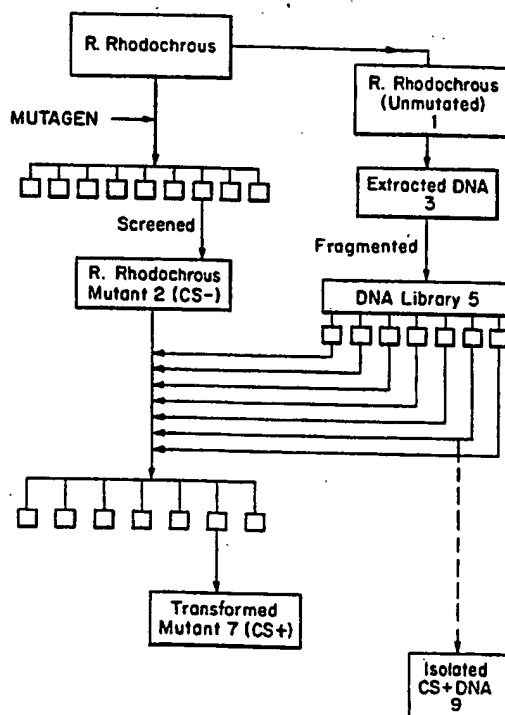
INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification <sup>5</sup> : C12N 15/52, C07K 15/00 C10G 32/00, C12S 1/02 C12N 1/21 // (C12N 1/21 C12R 1:01, 1:19)		A1	(11) International Publication Number: <b>WO 94/01563</b>
			(43) International Publication Date: 20 January 1994 (20.01.94)
(21) International Application Number: PCT/US93/06497 (22) International Filing Date: 9 July 1993 (09.07.93) (30) Priority data: 07/911,845                      10 July 1992 (10.07.92)                      US (60) Parent Application or Grant (63) Related by Continuation US    07/911,845 (CIP) Filed on                                      10 July 1992 (10.07.92)		(72) Inventors; and (75) Inventors/Applicants (for US only) : RAMBOSEK, John [US/US]; 7701 17th Avenue, NE, Seattle, WA 98115 (US). PIDDINGTON, Chris, S. [US/US]; 736 North 70th Street, Seattle, WA 98103 (US). KOVACEVICH, Brian, R. [US/US]; 316 Northeast 72nd Street, Seattle, WA 98115 (US). YOUNG, Kevin, D. [US/US]; 1102 Oak Street, Grand Forks, ND 58201 (US). DENOME, Sylvia, A. [US/US]; RR No. 1, Box 76c, Thompson, ND 58278 (US). (74) Agents: BROOK, David, E. et al.; Hamilton, Brook, Smith & Reynolds, Two Militia Drive, Lexington, MA 02173 (US). (81) Designated States: AT, AU, BB, BG, BR, BY, CA, CH, CZ, DE, DK, ES, FI, GB, HU, JP, KP, KR, KZ, LK, LU, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SK, UA, US, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.	
(71) Applicant (for all designated States except US): ENERGY BIOSYSTEMS CORPORATION [US/US]; 3608 Research Forest Drive, B7, The Woodlands, TX 77383 (US).			

(54) Title: RECOMBINANT DNA ENCODING A DESULFURIZATION BIOCATALYST

(57) Abstract

This invention relates to a recombinant DNA molecule containing a gene or genes which encode a biocatalyst capable of desulfurizing a fossil fuel which contains organic sulfur molecules. For example, the present invention encompasses a recombinant DNA molecule containing a gene or genes of a strain of *Rhodococcus rhodochrous*.



**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	FR	France	MR	Mauritania
AU	Australia	GA	Gabon	MW	Malawi
BB	Barbados	GB	United Kingdom	NE	Niger
BE	Belgium	GN	Guinea	NL	Netherlands
BF	Burkina Faso	GR	Greece	NO	Norway
BG	Bulgaria	HU	Hungary	NZ	New Zealand
BJ	Benin	IE	Ireland	PL	Poland
BR	Brazil	IT	Italy	PT	Portugal
BY	Belarus	JP	Japan	RO	Romania
CA	Canada	KP	Democratic People's Republic of Korea	RU	Russian Federation
CF	Central African Republic	KR	Republic of Korea	SD	Sudan
CG	Congo	KZ	Kazakhstan	SE	Sweden
CH	Switzerland	LI	Liechtenstein	SI	Slovenia
CI	Côte d'Ivoire	LK	Sri Lanka	SK	Slovak Republic
CM	Cameroon	LU	Luxembourg	SN	Senegal
CN	China	LV	Latvia	TD	Chad
CS	Czechoslovakia	MC	Monaco	TC	Togo
CZ	Czech Republic	MG	Madagascar	UA	Ukraine
DE	Germany	ML	Mali	US	United States of America
DK	Denmark	MN	Mongolia	UZ	Uzbekistan
ES	Spain			VN	Viet Nam
FI	Finland				

RECOMBINANT DNA ENCODING A DESULFURIZATION BIOCATALYSTBACKGROUND

Sulfur contaminants in fossil fuels can create problems in refinery processes which can be costly to rectify. The sulfur contaminants that occur in fossil fuels fall into either of the following general classes: mineralized (inorganic, e.g., pyritic) sulfur and organic sulfur (sulfur that is covalently bound to carbonaceous molecules, referred to as organosulfur compounds). The presence of sulfur has been correlated with corrosion of pipeline, pumping and refining equipment, and with premature breakdown of combustion engines. Sulfur also poisons many catalysts which are used in the refining of fossil fuels. Moreover, the atmospheric emission of sulfur combustion products, such as sulfur dioxide, leads to the form of acid deposition known as acid rain. Acid rain has lasting deleterious effects on aquatic and forest ecosystems, as well as on agricultural areas located downwind of combustion facilities. Monticello, D.J. and W.R. Finnerty, (1985) Ann. Rev. Microbiol. 39:371-389. Regulations such as the Clean Air Act of 1964 require the removal of sulfur, either pre- or post-combustion, from virtually all coal- and petroleum-based fuels. Conformity with such legislation has become increasingly problematic due to the rising need to utilize lower grade, higher-sulfur fossil fuels as clean-burning, low-sulfur petroleum reserves become depleted, as well as the progressive reductions in sulfur emissions required by regulatory authorities. Monticello, D.J. and J.J. Kilbane, "Practical Considerations in Biodesulfurization of Petroleum", IGT's 3d Intl. Symp. on Gas, Oil, Coal, and Env. Biotech., (Dec. 3-5, 1990) New Orleans, LA.

One technique which is currently employed for the pre-combustion removal of organic sulfur from liquid

-2-

fossil fuels, e.g., petroleum, is hydrodesulfurization (HDS). HDS is suitable for the desulfurization of fossil fuels wherein organosulfur compounds account for a significant, e.g., a major, proportion of all sulfur contaminants present. HDS is thus useful for treating crude oil or bitumen, petroleum distillate fractions or refining intermediates, liquid motor fuels, and the like. HDS is more particularly described in Shih, S.S. et al., "Deep Desulfurization of Distillate Components", Abstract No. 264B AIChE Chicago Annual Meeting, presented November 12, 1990, (complete text available upon request from the American Institute of Chemical Engineers); Gary, J.H. and G.E. Handwerk, (1975) Petroleum Refining: Technology and Economics, Marcel Dekker, Inc., New York, pp. 114-120, and Speight, J.G., (1981) The Desulfurization of Heavy Oils and Residue, Marcel Dekker, Inc., New York, pp. 119-127. HDS is based on the reductive conversion of organic sulfur into hydrogen sulfide ( $H_2S$ ) in the presence of a metal catalyst. HDS is carried out under conditions of elevated temperature and pressure. The hydrogen sulfide produced as a result of HDS is a corrosive gaseous substance, which is stripped from the fossil fuel by known techniques. Elevated or persistent levels of hydrogen sulfide are known to poison (inactivate) the HDS catalyst, complicating the desulfurization of liquid fossil fuels that are high in sulfur.

Organic sulfur in both coal and petroleum fossil fuels is present in a myriad of compounds, some of which are termed labile in that they can readily be desulfurized, others of which are termed refractory in that they do not easily yield to conventional desulfurization treatment, e.g., by HDS. Shih, S.S. et al. Frequently, then, even HDS-treated fossil fuels must be post-combustively desulfurized using an apparatus such as a flue scrubber. Flue scrubbers are expensive to install and difficult to maintain, especially for small combustion facilities.

-3-

Moreover, of the sulfur-generated problems noted above, the use of flue scrubbers in conjunction with HDS is directed to addressing environmental acid deposition, rather than other sulfur-associated problems, such as corrosion of machinery and poisoning of catalysts.

Recognizing these and other shortcomings of HDS, many investigators have pursued the development of microbial desulfurization (MDS). MDS is generally described as the harnessing of metabolic processes of suitable bacteria to the desulfurization of fossil fuels. Thus, MDS typically involves mild (e.g., ambient or physiological) conditions, and does not involve the extremes of temperature and pressure required for HDS. It is also generally considered advantageous that biological desulfurizing agents can renew or replenish themselves under suitable conditions. Microbial desulfurization technology is reviewed in Monticello and Finnerty (1985), 39 ANN. REV. MICROBIOL. 371-389 and Bhadra et al. (1987), 5 BIOTECH. ADV. 1-27. Hartdegan et al. (1984), 5 CHEM. ENG. PROGRESS 63-67 and Kilbane (1989), 7 TRENDS BIOTECHNOL. (No. 4) 97-101 provide additional commentary on developments in the field.

Several investigators have reported mutagenizing naturally-occurring bacteria into mutant strains with the acquired capability of breaking down, i.e., catabolizing, dibenzothiophene (DBT). Hartdegan, F.J. et al., (May 1984) Chem. Eng. Progress 63-67. DBT is representative of the class of organic sulfur molecules found in fossil fuels from which it is most difficult to remove sulfur by HDS. Most of the reported mutant microorganisms act upon DBT nonspecifically, by cleaving carbon-carbon bonds, thereby releasing sulfur in the form of small organic breakdown products. One consequence of this microbial action is that the fuel value of a fossil fuel so treated is degraded. Isbister and Doyle, however, reported the derivation of a mutant strain of Pseudomonas which appeared to be capable of selectively liberating sulfur from

-4-

DBT, thereby preserving the fuel value of treated fossil fuels. U.S. Patent No. 4,562,156.

Kilbane recently reported the mutagenesis of a mixed bacterial culture, producing a bacterial consortium which appeared capable of selectively liberating sulfur from DBT by an oxidative pathway. Resour. Cons. Recycl. 3:69-79 (1990). A strain of Rhodococcus rhodocrous was subsequently isolated from the consortium. This strain, which has been deposited with the American Type Culture Collection under the terms of the Budapest Treaty as ATCC No. 53968 and also referred to as IGTS8, is a source of biocatalytic activity as described herein. Microorganisms of the ATCC No. 53968 strain liberate sulfur from forms of organic sulfur known to be present in fossil fuels, including DBT, by the selective, oxidative cleavage of carbon-sulfur bonds in organic sulfur molecules. Kilbane has described the isolation and characteristics of this strain in detail in U.S. Patent No. 5,104,801.

#### SUMMARY OF THE INVENTION

This invention relates in one aspect to a deoxyribonucleic acid (DNA) molecule containing one or more genes encoding one or more enzymes that, singly or in concert with each other, act as a biocatalyst that desulfurizes a fossil fuel that contains organic sulfur molecules. The DNA molecule of the present invention can be purified and isolated from a natural source, or can be a fragment or portion of a recombinant DNA molecule that is, e.g., integrated into the genome of a non-human host organism. The gene or genes of the present invention can be obtained from, e.g., a strain of Rhodococcus rhodochrous microorganisms having suitable biocatalytic activity. That is, the gene or genes of the present invention can be obtained from a non-human organism, e.g., a microorganism, that expresses one or more enzymes that, singly or in concert with each other, act as a desulfurizing biocatalyst.

-5-

Biocatalysis, as described more fully below, is the selective oxidative cleavage of carbon-sulfur bonds in organosulfur compounds. The present invention is particularly useful for the desulfurization of fossil fuels that contain organosulfur compounds, e.g., DBT.

The invention further relates to recombinant DNA vectors, recombinant DNA plasmids and non-human organisms that contain foreign (recombinant, heterologous) DNA encoding a biocatalyst capable of desulfurizing a fossil fuel which contains organosulfur compounds. Such organisms are referred to herein as host organisms.

The invention described herein thus encompasses ribonucleic acid (RNA) transcripts of the gene or genes of the present invention, as well as polypeptide expression product(s) of the gene or genes of the present invention. The present polypeptide expression products, after such post-translational processing and/or folding as is necessary, and in conjunction with any coenzymes, cofactors or coreactants as are necessary, form one or more protein biocatalysts (enzymes) that, singly or in concert with each other, catalyze (promote, direct or facilitate) the removal of sulfur from organosulfur compounds that are found in fossil fuels. This is accomplished by the selective, oxidative cleavage of carbon-sulfur bonds in said compounds. The biocatalyst of the present invention can be a non-human host organism, viable (e.g., cultured) or non-viable (e.g., heat-killed) containing the DNA of the present invention and expressing one or more enzymes encoded therein, or it can be a cell-free preparation derived from said organism and containing said one or more biocatalytic enzymes.

In another aspect, the present invention relates to a method of desulfurizing a fossil fuel using the above mentioned non-human organism, said organism expressing a desulfurizing biocatalyst. Alternatively, the present invention relates to a method of desulfurizing a fossil

-6-

fuel using a biocatalyst preparation comprising one or more enzymes isolated from said organism. The process involves: 1) contacting said organism or biocatalyst preparation obtained therefrom with a fossil fuel that  
5 contains organic sulfur, such that a mixture is formed; and 2) incubating the mixture for a sufficient time for the biocatalyst expressed by or prepared from the organism to desulfurize the fossil fuel. The biocatalytically treated fossil fuel obtained following incubation has  
10 significantly reduced levels of organosulfur compounds, compared to a sample of the corresponding untreated fossil fuel.

In yet another aspect, the invention relates to nucleic acid probes which hybridize to the recombinant DNA  
15 of the present invention.

In still other aspects, the present invention relates to the production of new non-human organisms containing the recombinant DNA of the present invention and preferably expressing the biocatalyst encoded therein. Availability of the recombinant DNA of this invention greatly  
20 simplifies and facilitates the production and purification of biocatalysts for desulfurizing a fossil fuel. Costly and time consuming procedures involved in the purification of biocatalysts can be reduced, eliminating the need to  
25 generate the biocatalyst from one or more non-human organisms in which it is naturally present or has been produced by mutagenesis. More specifically, non-human host organisms can be generated which express the gene or genes of the present invention at elevated levels. In addition,  
30 the invention described herein furthers the discovery of genes encoding desulfurization biocatalysts in additional non-human organisms. This objective can be accomplished using the nucleic acid probes of the present invention to screen DNA libraries prepared from one or more additional  
35 non-human organisms in whom biocatalytic function is known or suspected to be present. Any genes present in such



-7-

organisms and encoding desulfurization biocatalysts or components thereof can be replicated at large scale using known techniques, such as polymerase chain reaction (PCR). PCR advantageously eliminates the need to grow the non-human organisms, e.g., in culture, in order to obtain large amounts of the DNA of interest.

#### BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a flow diagram schematic illustrating a stepwise procedure for the isolation of the recombinant DNA of the present invention.

Figure 2 is a schematic illustration of the Rhodococcus rhodochrous replication competent and chloramphenicol resistant vector pRF29, said vector having been derived from Rhodococcus fascians.

Figure 3 is a schematic illustration of the Rhodococcus rhodochrous replication competent and chloramphenicol resistant vector pRR-6.

Figure 4 is a schematic illustration of the restriction map for DNA plasmid pTOXI-1 encoding a biocatalyst capable of carbon-sulfur bond cleavage.

Figure 5 is a schematic illustration of the restriction map for subclone pMELV-1, derived from plasmid pTOXI-1.

Figure 6 is a schematic illustration of the restriction map for pMELV-1 and fragments thereof present as inserts in subclones pSMELV-1A, pSMELV-2A, pSMELV-3A and pSMELV-4A.

Figure 7 is a schematic illustration of the predicted locations within the sequence of pTOXI-1 of three nearly contiguous open reading frames (ORFs; specifically, ORF 1, ORF 2 and ORF 3) encoding polypeptide expression products responsible for the Dsz+ phenotype.

Figure 8 is a schematic illustration of the restriction map of pTOXI-1 and fragments thereof present as inserts in subclones pENOK-1, pENOK-2, pENOK-3, pENOK-11,

-8-

pENOK-13, pENOK-16, pENOK-18, pENOK-Nsi, pENOK-19 AND pENOK-20.

Figure 9 is a schematic illustration of the restriction map of pRR-12.

5        Figure 10 is a schematic illustration of the restriction map of vector pKAMI. In the inset, the engineered cloning site present in pKAMI is shown in detail.

Figure 11 is a schematic illustration of the restriction map of pSBG-2, in which expression of a promoterless  
10        Dsz gene cluster from pTOXI-1 is driven by the chloramphenicol resistance promoter.

#### DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

In the petroleum extraction and refining arts, the term "organic sulfur" is generally understood as referring  
15        to organic molecules having a hydrocarbon framework to which one or more sulfur atoms (called heteroatoms) are covalently joined. These sulfur atoms can be joined directly to the hydrocarbon framework, e.g., by one or more carbon-sulfur bonds, or can be present in a sub-  
20        stituent joined to the hydrocarbon framework of the molecule, e.g., a sulfonyl group (which contains a carbon-oxygen-sulfur covalent linkage). The general class of organic molecules having one or more sulfur heteroatoms are sometimes referred to as "organosulfur compounds".  
25        The hydrocarbon portion of these compounds can be aliphatic, aromatic, or partially aliphatic and partially aromatic.

Cyclic or condensed multicyclic organosulfur compounds in which one or more sulfur heteroatoms are linked  
30        to adjacent carbon atoms in the hydrocarbon framework by aromatic carbon-sulfur bonds are referred to as "sulfur-bearing heterocycles". The sulfur that is present in many types of sulfur-bearing heterocycles is referred to as "thiophenic sulfur" in vi w of the five-membered aromatic  
35        ring in which the sulfur heteroatom is present. The

-9-

simplest such sulfur-bearing heterocycle is thiophene, which has the composition  $C_4H_4S$ .

Sulfur-bearing heterocycles are known to be stable to conventional desulfurization treatments, such as HDS. For this reason, they are said to be refractory or recalcitrant to HDS treatment. Sulfur-bearing heterocycles can have relatively simple or relatively complex chemical structures. In complex heterocycles, multiple condensed aromatic rings, one or more of which can be heterocyclic, are present. The difficulty of desulfurization increases with the structural complexity of the molecule. Shih et al. That is, refractory behavior is most accentuated in complex sulfur-bearing heterocycles, such as dibenzothiophene (DBT,  $C_{12}H_8S$ ).

DBT is a sulfur-bearing heterocycle that has a condensed, multiple aromatic ring structure in which a five-membered thiophenic ring is flanked by two six-membered benzylic rings. Much of the residual post-HDS organic sulfur in fossil fuel refining intermediates and combustible products is thiophenic sulfur. The majority of this residual thiophenic sulfur is present in DBT and derivatives thereof having one or more alkyl or aryl radicals attached to one or more carbon atoms present in one or both flanking benzylic rings. Such DBT derivatives are said to be "decorated" with these radicals. DBT itself is accepted in the relevant arts as a model compound illustrative of the behavior of the class of compounds encompassing DBT and alkyl- and/or aryl-decorated derivatives thereof in reactions involving thiophenic sulfur. Monticello and Finnerty (1985), Microbial desulfurization of fossil fuels, 39 ANNUAL REVIEWS IN MICROBIOLOGY 371-389, at 372-373. DBT and radical-decorated derivatives thereof can account for a significant percentage of the total sulfur content of particular crude oils, coals and bitumen. For example, these sulfur-bearing heterocycles have been reported to account for as much

-10-

as 70 wt% of the total sulfur content of West Texas crude oil, and up to 40 wt% of the total sulfur content of some Middle East crude oils. Thus, DBT is considered to be particularly relevant as a model compound for the forms of thiophenic sulfur found in fossil fuels, such as crude oils, coals or bitumen of particular geographic origin, and various refining intermediates and fuel products manufactured therefrom. Id. Another characteristic of DBT and radical-decorated derivatives thereof is that, following a release of fossil fuel into the environment, these sulfur-bearing heterocycles persist for long periods of time without significant biodegradation. Gundlach et al. (1983), 221 SCIENCE 122-129. Thus, most prevalent naturally occurring microorganisms do not effectively metabolize and break down sulfur-bearing heterocycles.

A fossil fuel that is suitable for desulfurization treatment according to the present invention is one that contains organic sulfur. Such a fossil fuel is referred to as a "substrate fossil fuel". Substrate fossil fuels that are rich in thiophenic sulfur (wherein a significant fraction of the total organic sulfur is thiophenic sulfur, present in sulfur-bearing heterocycles, e.g., DBT) are particularly suitable for desulfurization according to the method described herein. Examples of such substrate fossil fuels include Cerro Negro or Orinoco heavy crude oils; Athabaskan tar and other types of bitumen; petroleum refining fractions such as light cycle oil, heavy atmospheric gas oil, and No. 1 diesel oil; and coal-derived liquids manufactured from sources such as Pocahontas #3, Lewis-Stock, Australian Glencoe or Wyodak coal.

Biocatalytic desulfurization (biocatalysis or BDS) is the excision (liberation or removal) of sulfur from organosulfur compounds, including refractory organosulfur compounds such as sulfur-bearing heterocycles, as a result of the selective, oxidative cleavage of carbon-sulfur bonds in said compounds by a biocatalyst. BDS treatment

-11-

yields the desulfurized combustible hydrocarbon framework of the former refractory organosulfur compound, along with inorganic sulfur -- substances which can be readily separated from each other by known techniques such as fractional distillation or water extraction. For example, DBT is converted into hydroxybiphenyl or dihydroxybiphenyl, or a mixture thereof, when subjected to BDS treatment. BDS is carried out by a biocatalyst comprising one or more non-human organisms (e.g., microorganisms) that functionally express one or more enzymes that direct, singly or in concert with each other, the removal of sulfur from organosulfur compounds, including sulfur-bearing heterocycles, by the selective cleavage of carbon-sulfur bonds in said compounds; one or more enzymes obtained from such microorganisms; or a mixture of such microorganisms and enzymes. Organisms that exhibit biocatalytic activity are referred to herein as being CS+ or Dsz+. Organisms that lack biocatalytic activity are referred to herein as being CS- or Dsz-.

As summarized above, the invention described herein relates in one aspect to a DNA molecule or fragment thereof containing a gene or genes which encode a biocatalyst capable of desulfurizing a fossil fuel that contains organosulfur compounds. The present DNA molecule or fragment thereof can be purified and isolated DNA obtained from, e.g., a natural source, or can be recombinant (heterologous or foreign) DNA that is, e.g., present in a non-human host organism. The following discussion, which is not to be construed as limiting on the invention in any way but is presented for purposes of illustration, recounts the isolation of DNA encoding a desulfurizing biocatalyst from a strain of Rhodococcus rhodochrous, ATCC No. 53968, that is known to express suitable biocatalytic activity. This preferred strain of Rhodococcus rhodochrous is disclosed in U.S. Patent No. 5,104,801 (issued 1992), the teachings of which are incorporated herein by refer-

-12-

ence, and has been referred to in the literature as IGTS8. IGTS8 was developed by investigators at the Institute of Gas Technology in Chicago IL. Other organisms that are known to express suitable biocatalytic activity and thus  
5 are viewed as suitable sources of the DNA of the present invention include the strain of Bacillus sulfasportare described in U.S. Patent 5,002,888 and deposited with the American Type Culture Collection as ATCC No. 53969 and the Corynebacterium strain described in Omori et al. (1992),  
10 Desulfurization of dibenzothiophene by Corynebacterium sp. strain SY1, 58 APPL. ENV. MICROBIOL. (No. 3) 911-915. The isolation of the DNA of the present invention from the ATCC No. 53968 microorganism is schematically depicted in Figure 1, and will now be described.

15 Mutant strains of R. rhodochrous that are incapable of cleaving carbon-sulfur bonds (CS- or Dsz-), are produced by exposing a strain of R. rhodochrous, e.g., ATCC No. 53968, that exhibits biocatalytic activity (that is CS+ or Dsz+), to a mutagen under appropriate conditions  
20 that are known to or readily ascertainable by those skilled in the art. Suitable mutagens include radiation, e.g., ultraviolet radiation, and chemical mutagens, e.g., N-methyl-N'-nitrosoguanidine (NTG), hydroxylamine, ethylmethanesulphonate (EMS) and nitrous acid. Mutants thus  
25 formed are allowed to grow in an appropriate medium and screened for carbon-sulfur bond cleavage activity. Mutants identified as lacking carbon-sulfur bond cleavage activity are termed CS-. Any method of screening which allows for an accurate detection of carbon-sulfur bond  
30 cleavage activity is suitable in the method of the present invention. Suitable methods of screening for this activity include exposing the different mutants to carbon-sulfur bond containing molecules (e.g., DBT) and measuring carbon-sulfur bond cleavage. In a preferred embodiment,  
35 the mutants are exposed to DBT, such that the breakdown product, hydroxybiphenyl (HBP), which fluoresces under

-13-

short wave ultraviolet light, is produced. HBP can also be detected colorimetrically through its blue reaction product with Gibbs' reagent. Other methods include gas and liquid chromatography, infrared and nuclear magnetic resonance spectrometry. See Kodama, et al., Applied and Environmental Microbiology, pages 911-915 (1992) and Kilbane and Bielaga, Final Report D.O.E. Contract No. DE-AC22-88PC8891 (1991). Once CS- mutants are identified and isolated, clones thereof are propagated using standard techniques and subjected to further analysis.

Concurrent with the mutagenesis of the above-described culture of the CS+ organism, R. rhodochrous, a second culture of the same CS+ organism (1) is maintained in culture. CS+ organism DNA (3) is extracted from this culture of R. rhodochrous. Various methods of DNA extraction are suitable for isolating the DNA of this organism. Suitable methods include phenol and chloroform extraction. See Maniatis et al., Molecular Cloning, A Laboratory Manual, 2d, Cold Spring Harbor Laboratory Press, page 16.54 (1989), herein referred to as Maniatis et al..

Once the DNA is extracted from R. rhodochrous 1, the DNA (3) is cut into fragments of various kilobase lengths, which collectively make up DNA library 5. Various methods of fragmenting the DNA of R. rhodochrous to purify DNA therefrom, including the DNA of the present invention, can be used, e.g., enzymatic and mechanical methods. Any four-base recognition restriction endonuclease such as TaqI or Sau 3A is suitable for fragmenting the DNA. Suitable methods of fragmenting DNA can be found in Maniatis et al..

The various DNA fragments are inserted into several CS- mutant clones of R. rhodochrous (2), with the purpose of isolating the fragment of DNA that encodes the biocatalyst of the present invention. The transformation of a previously CS- mutant cell to a CS+ transformed cell is evidence that the inserted DNA fragment encodes a bio-

-14-

catalyst. Any method of inserting DNA into R. rhodochrous which allows for the uptake and expression of said fragment is suitable. In a preferred embodiment, electroporation is used to introduce the DNA fragment into R. rhodochrous. See Maniatis et al..

Once transformed, CS+ mutant R. rhodochrous 7 has been produced and identified, DNA fragment 9 encoding the CS+ biocatalyst can be identified and isolated. The encoded biocatalyst can then be produced using the isolated DNA in various methods that are well known and readily available to those skilled in the art. In addition, the isolated DNA can be sequenced and replicated by known techniques, e.g., the techniques described in Maniatis et al..

As noted previously, the above-described method for isolating the DNA of the present invention can be applied to CS+ organisms other than R. rhodochrous microorganisms, e.g., of the strain ATCC No. 53968. Thus, Bacillus subtilis ATCC No. 53969 or Corynebacterium sp. SY1 can be used as the source organism for the DNA of the present invention. Furthermore, once isolated, the DNA of the present invention can be transfected into a non-human host organism other than a CS- mutant of the source organism. Thus, the DNA of the present invention can be transfected into, e.g., a suitable strain of Escherichia coli bacteria. Other types of non-human host organism can also be used, including unicellular organisms (e.g., yeast) and cells established in culture from multicellular organisms.

Other methods of isolating the DNA of the present invention, include variations on the rationale described above and depicted in Figure 1. For example, it would be possible to randomly insert a CS- DNA plasmid into clones of a CS+ strain of R. rhodochrous. DNA encoding a CS+ biocatalyst could then be identified by screening for clones that have been transformed from CS+ to CS-.



-15-

The recombinant DNA molecule or fragment thereof of the present invention is intended to encompass any DNA resulting from the insertion into its chain, by chemical or biological means, of one or more genes encoding a biocatalyst capable of selectively cleaving carbon-sulfur bonds, said gene not originally present in that chain. Recombinant DNA includes any DNA created by procedures using restriction nucleases, nucleic acid hybridization, DNA cloning, DNA sequencing or any combination of the preceding. Methods of construction can be found in Maniatis et al., and in other methods known by those skilled in the art.

Procedures for the construction of the DNA plasmids or vectors of the present invention include those described in Maniatis et al. and other methods known by those skilled in the art. Suitable plasmid vectors include pRF-29 and pRR-6 depicted in Figures 2 and 3, respectively. The terms "DNA plasmid" and "vector" are intended to encompass any replication competent plasmid or vector capable of having foreign or exogenous DNA inserted into it by chemical or biological means and subsequently, when transfected into an appropriate non-human host organism, of expressing the product of the foreign or exogenous DNA insert (i.e., of expressing the biocatalyst of the present invention). In addition, the plasmid or vector must be receptive to the insertion of a DNA molecule or fragment thereof containing the gene or genes of the present invention, said gene or genes encoding a biocatalyst that selectively cleaves carbon-sulfur bonds in organosulfur compounds. Procedures for the construction of DNA plasmid vectors include those described in Maniatis et al. and others known by those skilled in the art.

The plasmids of the present invention include any DNA fragment containing a gene or genes encoding a biocatalyst that selectively cleaves carbon-sulfur bonds in organosulfur compounds. The term "plasmid" is intended to

-16-

encompass any DNA fragment. The DNA fragment should be transmittable to a host microorganism by transformation or conjugation. Procedures for the construction or extraction of DNA plasmids include those described in Maniatis  
5 et al. and others known by those skilled in the art.

The transformed non-human host organisms of the present invention can be created by various methods by those skilled in the art. For example, transfection electroporation as explained by Maniatis et al. can be  
10 used. By the term "non-human host organism" is intended any non-human organism capable of the uptake and expression of foreign, exogenous or recombinant DNA, i.e., DNA not originally a part of the organism's nuclear material.

The method of desulfurizing a fossil fuel of the  
15 present invention involves two aspects. First, a host organism or biocatalytic preparation obtained therefrom is contacted with a fossil fuel to be desulfurized. This can be done in any appropriate container, optionally fitted with an agitation or mixing device. The mixture is com-  
20 bined thoroughly and allowed to incubate for a sufficient time to allow for cleavage of a significant number of carbon-sulfur bonds in organosulfur compounds, thereby producing a desulfurized fossil fuel. In one embodiment, an aqueous emulsion is produced with an aqueous culture of  
25 the organism and the fossil fuel, allowing the organism to propagate in the emulsion while the expressed biocatalyst cleaves carbon-sulfur bonds.

Variables such as temperature, mixing rate and rate of desulfurization will vary according to the organism  
30 used. The parameters can be determined through no more than routine experimentation.

Several suitable techniques for monitoring the rate and extent of desulfurization are well-known and readily available to those skilled in the art. Baseline and  
35 timecourse samples can be collected from the incubation mixture, and prepared for a determination of the residual

-17-

organic sulfur in the fossil fuel. The disappearance of sulfur from organosulfur compounds, such as DBT, in the sample being subjected to biocatalytic treatment can be monitored using, e.g., X-ray fluorescence (XRF) or atomic emission spectrometry (flame spectrometry). Preferably, the molecular components of the sample are first separated, e.g., by gas chromatography.

The nucleic acid probes of the present invention include any nuclear material capable of hybridizing to at least a portion of the DNA of the present invention. The term "nucleic acid probe" includes any nuclear material capable of hybridizing to DNA.

The invention will now be further illustrated by the following specific Examples, which are not to be viewed as limiting in any way.

EXAMPLE 1. ISOLATION OF DNA ENCODING A DESULFURIZATION ACTIVE BIOCATALYST.

As used herein, the term "Dsz+" refers to the ability of an organism to utilize thiophenic compounds such as dibenzothiophene (DBT) as the sole source of sulfur by the selective cleavage of carbon-sulfur bonds therein. Rhodococcus rhodochrous strain IGTS8 demonstrates the Dsz<sup>+</sup> phenotype. The term "Dsz-" refers to an organism's inability to utilize said thiophenic compounds as a sole source of sulfur by the selective cleavage of carbon-sulfur bonds therein.

**Materials**

Bacterial strains and plasmids

Rhodococcus rhodochrous strain IGTS8 (ATCC No. 53968), obtained from the Institute of Gas Technology (Chicago, IL), was used as a parent strain for production of mutant strains which have lost the desulfurization phenotype (Dsz-). Strain IGTS8 was also used for isolation of DNA fragments capable of complementing said

-18-

mutants to produce Dsz+ mutants therefrom. Rhodococcus vector pRF-29 was obtained from the Institute of Gas Technology. The construction of pRF-29 is described in Desomer, et al. (1990), Transformation of Rhodococcus  
5 fascians by High-Voltage Electroporation and Development of R. fascians Cloning Vectors, APPLIED AND ENVIRONMENTAL MICROBIOLOGY 2818-2825. The structure of pRF-29 is schematically depicted in Figure 2.

Escherichia coli strain JM109 was used as a host in  
10 transformation with plasmid constructs derived from the plasmids pUC18 and pUC19 (Bethesda Research Laboratories, Bethesda, MD).

#### Enzymes and Reagents

Restriction endonucleases were purchased from  
15 Bethesda Research Laboratories (BRL) and New England Biolabs (Beverly, MA). T4 ligase and the Klenow fragment of E. coli DNA polymerase I were purchased from BRL. HK<sup>TM</sup> Phosphatase was purchased from Epicentre Technologies (Madison, WI). All enzymes were used in accordance with  
20 manufacturers recommendations. Enzyme assay substrates Dibenzothiophene (DBT), Dibenzothiophene 5-oxide (DBT sulfoxide) and Dibenzothiophene sulfone (DBT sulfone) were purchased from Aldrich (Milwaukee, WI). Gibb's Reagent, 2,6-dichloroquinone-4-chloroimide, was purchased from  
25 Sigma (St. Louis, MO). Chemical mutagen N-methyl-N'-nitro-N-nitrosoguanidine (NTG) was also purchased from Sigma.

#### Growth Media and Conditions

E. coli JM109 was grown in L-broth (Difco, Detroit,  
30 MI). Transformants were selected on L-plates supplemented with 1.5% agar and containing 125µg/ml ampicillin. E. coli strains were grown at 37°C. Rhodococcus strains were maintained on Rhodococcus Media (RM) composed per liter of: 8.0g Nutrient Broth (Difco), 0.5g yeast extract, 10.0g

-19-

glucose. Transformants of Rhodococcus strains were selected on RM plates supplemented with 1.5% agar and containing 25µg/ml chloramphenicol. For expression of the Dsz+ phenotype, Rhodococcus strains were grown in Basal Salts Media (BSM) composed per liter of: 2.44g  $\text{KH}_2\text{PO}_4$ , 5.57g  $\text{Na}_2\text{HPO}_4$ , 2.0g  $\text{NH}_4\text{Cl}$ , 0.2 g  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ , 0.001g  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 0.001g  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ , 0.004g  $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ , 6.4ml glycerol. Optionally, BSM can be supplemented with 5.0g/liter glucose. Rhodococcus strains were grown at 30°C.

## Methods

### Sulfur Bioavailability Assay

The sulfur bioavailability assay, described in U.S. Patent 5,104,801, examines an organism's ability to liberate organically bound sulfur from substrates (e.g., DBT, DBT sulfoxide, DBT sulfone) for use as the sole source of sulfur for growth. In the assay, BSM, which contains no sulfur, is supplemented with one or more sulfur containing substrates, e.g., DBT. The organism's ability to liberate sulfur therefrom is measured by its ability to grow with proper incubation, as monitored by optical density at 600 nm.

### Gibbs Assay for 2-Hydroxybiphenyl

The oxidative product of DBT, DBT sulfoxide and DBT sulfone incubated with strain IGTS8 is 2-hydroxybiphenyl (2-HBP). The Gibbs assay colorimetrically quantitates the amount of 2-HBP produced from DBT and its above-mentioned oxidative derivatives. The assay measures 2-HBP produced in culture supernatants after incubation with DBT. The media must be adjusted to pH 8.0 before the Gibb's reagent is added. Gibb's R agent, 2,6- dichloroquinone-4-chloroimide (10mg/ml in ethanol), is added to culture supernatants at 1:100 (v/v). Color development is measured as

-20-

absorbance at 610nm after a 30 minute incubation at room temperature.

#### HPLC Assay for 2-Hydroxybiphenyl

2-HBP production cultures incubated with DBT can also  
5 be detected by HPLC using instrumentation available from  
Waters, Millipore Corporation, Milford, MA. Reagent  
alcohol is added to culture broth at 1:1 (v/v) in order to  
solubilize all remaining DBT and 2-HBP. Samples are  
agitated for 5 min at 220 rpm. Extracted broth samples  
10 are removed and centrifuged to remove cellular mass.  
Clarified supernatants are then analyzed by HPLC with the  
following conditions:

	Column:	Waters 4 $\mu$ Phenyl Novapak	
15	Detection Parameters:	DBT 2-HBP	233nm, 1.0 AUFS 248nm, 0.2 AUFS
	Quantitative Detection Limits:	DBT 2-HBP	10 - 250 $\mu$ M 6 - 60 $\mu$ M
20	Mobile Phase:	Isocratic 70% Acetonitrile 1.5ml/min	
	Retention times:	DBT 2-HBP	4.5 minutes 2.9 minutes

#### IGTS8 Mutagenesis

25 In order to generate mutant strains of R. rhodochrous  
which did not metabolize DBT (Dsz- mutants), biocatalyst  
source strain IGTS8 (Dsz+) was subjected to mutagenesis by  
short-wave UV light and to chemical mutagenesis with N-  
methyl-N'-nitro-N-nitrosoguanidine (NTG). With UV ex-  
30 posure mutagenesis, a kill rate of greater than 99% was  
targeted. Continuously stirred R. rhodochrous cells at an  
optical density ( $A_{660}$ ) of 0.3 were subjected to UV exposure  
from a Min ralight Lamp Model UVG-254 (Ultra-violet Pro-

-21-

ducts, Inc., San Gabriel, CA) at a distance of 10 cm for 55 to 65 seconds to obtain this kill rate (97.9-99.9%). For NTG mutagenesis, cell suspensions were treated with 500 µg/ml NTG for a duration determined to achieve a kill rate of 30%-50%. Combination mutagenesis utilizing both NTG and UV was also done. For these an overall kill rate of greater than 99.9% was used. Colonies surviving mutagenesis were picked onto RM plates and screened for the Dsz- phenotype as described below.

- 10 Screening Example A: Initially, a DBT-spray plate screen was used to select Dsz- mutants. Mutant colonies were replica plated onto Basal Salts Media (BSM) electrophoretic-grade agarose plates which contained no added sulfur. Colonies were allowed to grow at 30°C for 24hr.
- 15 The plates were then sprayed with an even coating of 10% DBT dissolved in ether and incubated at 30°C for 90 minutes. The plates were then wiped clean and observed under short-wave UV light. The observed end product of DBT metabolism, 2-hydroxybiphenyl (2-HBP) fluoresces under
- 20 short-wave UV light. Colonies that produce 2-HBP are thus identified by fluorescent spots on the agarose. Colonies that do not produce 2-HBP (that are Dsz-) do not produce fluorescent spots.

- Screening Example B: A simpler variation of screening
- 25 involved replica plating surviving mutagenized colonies to BSM agarose plates supplemented with 1.2ml/liter of a saturated ethanol solution of DBT. After 24 hours, production of 2-HBP can be visualized under UV illumination as above.

- 30 Mutants which did not appear to produce 2-HBP by the above-described screening methods were examined with the sulfur bioavailability assay, with DBT as the sole source of sulfur. Growth of potential mutants was examined in

-22-

1.25ml liquid fermentations in BSM plus DBT media dispensed in 24-well plates (Falcon). After a one day incubation at 30°C, 2-HBP production was monitored by the Gibbs colorimetric assay. Strains which continue to demonstrate the Dsz- phenotype were incubated in larger volumes of BSM plus DBT and analyzed for 2-HBP or intermediates by the HPLC method. Because BSM is a defined minimal medium, a duplicate control culture which contained supplemental inorganic sulfur was grown in order to distinguish true Dsz- mutants from auxotrophic mutants. Mutants which failed to grow in both the control and experimental media were assumed to be auxotrophic mutants.

Of 1970 individually analyzed potential mutants, two were identified as Dsz-. One mutant, GPE-362, was generated by NTG mutagenesis. The other, CPE-648, was generated by combination NTG/UV mutagenesis. Both GPE-362 and CPE-648 grow slowly in sulfur bioavailability assays, presumably from trace amounts of sulfur on the glassware or in the media components. However, no detectable amounts of 2-HBP were produced by either mutant after an extended incubation of 6 to 10 days with DBT, as assessed with either the Gibbs assay or the HPLC assay. Thus, independently produced R. rhodocrous IGTS8 mutants GPE-362 and CPE-648 were Dsz- organisms.

## 25 Vector Construction

Vector constructs were derived from R. rhodochrous and confer chloramphenicol resistance. All constructs were developed in E. coli strain JM109. Transformation of JM109 was carried out with the Gene Pulsar (Bio-Rad Laboratories, Richmond, CA) according to manufacturer's recommendations. Plasmid isolation from JM109 was performed by standard methods (Birnboim and Doly (1979), A rapid alkaline extraction procedure for screening recombinant plasmid DNA, 7 NUCLEIC ACIDS RES. 1513-1523; Maniatis et al. (1982), MOLECULAR CLONING: A LABORATORY MANUAL (Cold



-23-

Spring Harbor Laboratory Press). Transformants containing correct vector constructs were identified by restriction analysis.

Vector Construct A: pRR-6 (Figure 3) contains the Rhodo-  
5 coccus origin of replication and Chloramphenicol resistance marker ( $\text{Cm}^R$ ). The ori and  $\text{Cm}^R$  have been removed from pRF-29 as a 6.9kb XhoI/XbaI (partial) fragment. The ends were made blunt with Klenow and ligated to SmaII/XbaI cut pKF39. pKF39 is pUC18 with the SmaII site replaced  
10 with a BgIII site. A unique NarI site is available for cloning in pRR-6. NarI ends are compatible with 4-base recognition endonuclease TaqI.

#### Transformation of Rhodococcus rhodochrous

Transformation of IGTS8 and Dsz- mutants thereof can  
15 be achieved by electroporation. The following conditions were used in all transformations of Rhodococcus rhodochrous. Cells were grown in RM to mid-log phase and harvested by centrifugation (5000xg), then washed three times in cold, deionized, distilled water and concentrated  
20 50-fold in 10% glycerol. The resulting cell concentrate could be used for electroporation directly or stored at -80°C.

Electroporations were carried out with the Gene  
Pulser (Bio-Rad) apparatus. 100  $\mu\text{l}$  cells were mixed with  
25 transformation DNA in a 2-mm gapped electrocuvette (Bio-Rad) and subjected to a 2.5 kV pulse via the pulse controller (25  $\mu\text{F}$  capacitor, 200  $\Omega$  external resistance). Pulsed cells were mixed with 400  $\mu\text{l}$  RM and incubated for 4 hours at 30°C with regular agitation. Cells were then  
30 plated to RM supplemented with proper antibiotic.

When IGTS8 was transformed with pRF-29, chloramphenicol resistant colonies were cleanly selected at a frequency of  $10^5 - 10^6/\mu\text{g}$  DNA on plates containing 25  $\mu\text{g/ml}$  chloramphenicol.

-24-

Small Scale Plasmid Preparation from *R. rhodochrous*

A single colony of *Rhodococcus rhodochrous* was used to inoculate 2 to 7ml of RM plus 25 µg/ml chloramphenicol. The culture was incubated for two days at 30°C with shaking. Cells were pelleted by centrifugation and resuspended in 300 µl sucrose buffer (20% sucrose, 0.05 M Tris-Cl pH 8.0, 0.01 M EDTA 0.05 M NaCl, 10 mg/ml lysozyme) and incubated at 37°C for 1 hour. 300 µl Potassium acetate-acetate solution, pH 4.8 (60 ml 5 M KOAc, 11.5 ml Glacial acetic acid, 28.5 ml dH<sub>2</sub>O), was added and the mixture was gently mixed by inversion. The mixture was placed on ice for 5 minutes and then cellular debris was pelleted by centrifugation. 500 µl supernatant was removed to a fresh tube to which RNase was added to 0.05 µg/µl and incubated for 20 minutes at 37°C. The sample was then phenol:-chloroform extracted and the aqueous layer was precipitated at -80°C with an equal volume of isopropanol. DNA was pelleted by centrifugation and resuspended in 0.3 M NaOAc pH 8.0. DNA was precipitated again at -80°C with an equal volume of isopropanol. DNA was pelleted by centrifugation and resuspended in 0.3 M NaOAc pH 8.0. DNA was precipitated again at -80°C with two volumes of 95% EtOH. Pelleted DNA was washed with 70% EtOH and resuspended in 50 µl TE (Tris EDTA).

25 Isolation of Genomic DNA from *R. rhodochrous* Strain IGTS8

IGTS8 genomic DNA was isolated as described. 20 ml RM was inoculated with a single colony of IGTS8 and incubated at 30°C for 48 hours with shaking at 220 rpm. Cells were harvested by centrifugation (5000xg). Cells were resuspended in 10ml TE (10 mM Tris Base, 1 mM EDTA) with 100 mg lysozyme and incubated for 30 minutes at 30°C. Cells were lysed by adding 1 ml of 20% sodium dodecyl sulfate (SDS). 10 ml of TE-saturated phenol and 1.5 ml 5 M NaCl were added immediately and the mixture was gently agitated for 20 minutes at room temperature. Phenol was

-25-

removed by centrifugation, and the aqueous layer was extracted twice with an equal volume of chloroform. An equal volume of isopropanol was added to the aqueous layer to precipitate the DNA. DNA was spooled onto a pasteur  
5 pipette and redissolved in TE. DNA was then RNased with 20 µg/ml RNA for 1 hour at 37°C. The sample was made to a final concentration of 100 mM NaCl and 0.4% SDS and proteased with 100 µg/ml protease K. The sample was then extracted with phenol and chloroform and precipitated with  
10 isopropanol as before. The purified genomic DNA, which included the DNA of the present invention, was resuspended in TE.

#### Construction of Plasmid Library of IGTS8

Genomic DNA from the Dsz<sup>+</sup> source organism (IGTS8) was  
15 cut with TaqI in order to produce fragments 0.5 - 23 kb in length. Cut DNA was electrophoresed through 0.8% low melting temperature agarose and DNA fragments greater than 5 kb in length were isolated and purified by standard methods (Maniatis, T. et al. (1982), MOLECULAR CLONING: A  
20 LABORATORY MANUAL (Cold Spring Harbor Laboratory Press)). Vector pRR-6 was cut with NarI to completion. The vector ends were dephosphorylated with HK<sup>TM</sup> phosphatase to prevent religation of the vector. The size-fractioning genomic DNA was ligated to cut and dephosphorylated pRR-6.

#### 25 Molecular Complementation of Dsz<sup>-</sup> Mutant Strain CPE-648

Plasmid library ligations (above) were used to transform Dsz<sup>-</sup> mutant strain CPE-648 by electroporation as described. Negative control transformations of CPE-648, which did not contain DNA (mock transformations), were  
30 also performed. After the four hour incubation in RM, the cells were spun out of suspension by centrifugation and the supernatant was removed. The cells were resuspended in BSM with no sulfur. These cells were used to inoculate 250 ml of BSM supplemented with 300 µl of a saturated

-26-

ethanol solution of DBT. By this procedure, clones which are capable of complementing the Dsz- mutation will be selected by the sulfur bioavailability assay. Strains containing the complementing sequences (i.e., the DNA of the present invention) will successfully remove the sulfur from DBT and grow preferentially.

After 6 days incubation at 30°C, the cultures were assayed for 2-HBP by HPLC. Accumulation of 2-HBP was detected in experimental cultures while no accumulation of 2-HBP was detected in control cultures. The culture producing 2-HBP was spread onto RM plates supplemented with chloramphenicol to obtain single colonies that were harboring plasmids. These plates were replica-plated to BSM agarose plates supplemented with 1.2 ml/liter of a saturated ethanol solution of DBT. After 24 hours incubation at 30°C, 2-HBP could be detected around some individual colonies under short wave UV illumination. These colonies presumably harbored plasmids which complemented the Dsz- mutant by restoring the former Dsz+ phenotype.

#### Characterization of Clones Complementing Dsz- Mutant CPE-648

Two independent plasmid libraries successfully complemented mutant CPE-648 to Dsz<sup>+</sup> as described above. Plasmid DNA was isolated from single colonies which demonstrated 2-HBP production on BSM plus DBT plates (above) from cultures transformed with each of the two libraries. This plasmid DNA was used to transform *E. coli* strain JM109. Plasmid DNA was isolated and cut with restriction endonucleases in order to build a restriction map of the clones. Each of the two libraries yielded a single complementing clone. By restriction pattern similarities, the two clones appear to have overlapping sequences. These clones have been designated pTOXI-1 (Figure 4) and pTOXI-2, respectively. pTOXI-1 contains an insert of

-27-

approximately 6.6kb. pTOXI-2 contains an insert of approximately 16.8kb.

#### Complementation of Dsz<sup>-</sup> Mutant GPE-362

Dsz<sup>-</sup> mutant GPE-362 was transformed with plasmids pTOXI-1 and pTOXI-2. As a control, GPE-362 was also transformed with vector pRR-6. Transformants containing plasmid DNA were selected on RM plus chloramphenicol plates. Cm<sup>R</sup> colonies were transferred to BSM agarose plates supplemented with DBT. After 24 hr. incubation at 30°C, 2-HBP production could be seen around colonies containing either pTOXI-1 or pTOXI-2 by short wave UV illumination. No 2-HBP could be detected around colonies containing only vector pRR-6.

#### Overexpression of the Dsz<sup>+</sup> Trait Upon Reintroduction of Cloned DNA

Plasmids pTOXI-1 and pTOXI-2 were transformed into Dsz<sup>-</sup> mutant strain CPE-648. Transformants containing plasmid DNA were selected on RM plus chloramphenicol plates. The specific activity of individual clones was examined by the following protocol.

Single colonies of CPE-648 containing either pTOXI-1 or pTOXI-2 were used to inoculate 25 ml RM plus 25 µg/ml chloramphenicol in a 250 ml flask. As a positive control, parent strain IGTS8 was also grown in 25 ml RM. After 48 hours of growth at 30°C, 225 rpm shaking, 2.5 ml of the cultures were crossed into 25 ml BSM supplemented with 0.7 mM DMSO. Cultures were incubated for an additional 40 hours at 30°C. The optical density of each culture was measured at 600 nm against an appropriate blank. DBT was added to a final concentration of 150 µM and the cultures were incubated for 3 hours at 30°C. An equal volume of Reagent Alcohol (Baxter, McGaw Park, IL) was then added to each culture to solubilize any remaining DBT or 2-HBP. A 1 ml sample was removed and cellular debris removed by

-28-

centrifugation. The supernatant was analyzed for 2-HBP by the HPLC assay described above. The specific activity is calculated as mg of 2-HBP per liter/hours of incubation/OD<sub>600</sub>. The results of the above assay is listed in Table 1.

TABLE 1: Biocatalytic Desulfurization Activity of Transformed Mutants

STRAIN	OD <sub>600</sub>	2-HBP (mg/l)	Specific Activity (mg/l/hr/OD <sub>600</sub> )
IGTS8	2.89	3.94	0.45
GPE-362	1.53	0.00	0.00
CPE-648	4.10	0.00	0.00
CPE648 (pTOXI-1)	3.84	15.84	1.37
CPE648 (pTOXI-2)	2.88	5.74	0.66

10

15

EXAMPLE 2: DNA SEQUENCING OF A DESULFURIZATION ACTIVE BIOCATALYST BY THE DIDEOXY METHOD FROM PLASMID PTOXI-1

#### Materials

##### Bacterial strains and plasmids

Plasmid pTOXI-1 was used as the original source of DNA for sequencing. Escherichia coli strain JM109 was used as a host for subcloning and plasmid maintenance. Plasmids pUC18 and pUC19 were purchased from Bethesda Research Laboratories (Bethesda, MD).

20

##### Enzymes and Reagents

Restriction endonucleases were purchased from Beth sda Research Laboratories (BRL) and New England Biolabs (Beverly, MA). T4 ligase was purchased from BRL. A

-29-

Sequenase Version 2.0 DNA sequencing kit was purchased from United States Biochemical Corporation (Cleveland, OH). All enzymes and kits were used in accordance with manufacturer's recommendations.

## 5 Growth Media and conditions

E. coli strain JM109 harboring plasmids was grown in L-broth (Difco) containing 100 µg/ml ampicillin. Transformants were selected on L-plates supplemented with 1.5% agar and containing 100 µg/ml ampicillin. E. coli strains  
10 were grown at 37°C.

## Methods

### Plasmid DNA preparation from E. coli

Plasmid DNA was prepared from E. coli via lysis by SDS (Maniatis, et al.). The DNA was further purified  
15 through a polyethylene glycol precipitation before use in sequencing reactions.

### Plasmid Subcloning

The following subclones of pTOXI-1 were generated by standard techniques to aid in DNA sequencing:

20 a) pMELV-1 (Figure 5) was derived by isolating the 6.7kb HindIII/NdeI fragment from pTOXI-1 (shown in Figure 4) and ligating it to pUC-18 cut with HindIII/NdeI. JM109 cells harboring pMELV-1 were identified by plasmid isolation and restriction endonuclease analysis (Maniatis, et al.).  
25 al.).

b) pSMELV-1A (Figure 6) contains the 1.6kb SphI/XhoI fragment of pMELV-1 subcloned into pUC-18.

c) pSMELV-2A (Figure 6) contains the 0.7kb BamHI/SacI fragment of pMELV-1 subcloned into pUC-18.

30 d) pSMELV-3A (Figure 6) contains the 3.5kb SacI/XhoI fragment of pMELV-1 subcloned into pUC-18.

e) pSMELV-4A (Figure 6) contains the 1.5kb SphI/BamHI fragment of pMELV-1 subcloned into pUC-18.

-30-

Dideoxy Sequencing from Plasmid DNA

- a) Denaturation. Prior to sequencing reactions, plasmid DNA must be denatured. This was accomplished by treatment with NaOH. The denatured DNA is then recovered by addition of salt and EtOH precipitation. Preferably, 2-5 µg of denatured plasmid DNA is used in each sequencing reaction. See manufacturer's recommendations with Sequenase Version 2.0 DNA sequencing kit (United States Biochemical Corporation).
- b) Dideoxy sequencing. Chain termination dideoxy sequencing with Sequenase 2.0 was performed as described by the manufacturer (U.S. Biochemical Corporation). Sequencing of the cluster was initiated by priming sub-clones pMELV-1A, pMELV-2A, pMELV-3A, pMELV-4A with the "40 Universal Primer" defined as: 5'-GTTTTCCAGTCACGAC-3' and the "Reverse Primer" defined as: 5'-AACAGCTATGACCATG-3'. The sequence was extended by synthesizing overlapping oligonucleotides to previously read sequence using the Gene Assembler Plus (Pharmacia, Piscataway, NJ). The synthesized oligonucleotides were used as primers for continuing sequence reactions. Plasmid pMELV-1 was used as the template for all of the remaining sequences. DNA sequence was read from both strands of the plasmid clone to increase fidelity.

- EXAMPLE 3: COMPLEMENTATION CLONING OF A DESULFURIZATION ACTIVE BIOCATALYST FROM A COSMID LIBRARY; TRANSFECTION OF BIOCATALYST DNA INTO AN R. FASCIANS HOST ORGANISM**

Materials and Methods

- Bacterial strains, media and reagents

Rhodococcus sp. Rhodococcus rhodochrous strain IGTS8, obtained from the Institute of Gas Technology (Chicago,



-31-

IL) was used. UV1 is a mutant of IGTS8 that is unable to desulfurize DBT, described herein. R. fascians D188-5 (Desomer, et al., J. Bacteriol., 170:2401-2405, 1988) and R. rhodochrous ATCC13808 (type strain from ATCC) do not metabolize DBT. E. coli XL1-Blue (from Stratagene Cloning System, La Jolla, CA) is recA1 lac thi endA1 gyrA96 hsdR17 supE44 relA1 [F' proAB lacI<sup>q</sup> lacZ4M15 Tn10]. E. coli CS109 is W1485 thi supE F'. E. coli S17-1 is a derivative of E. coli 294 and is recA thi pro hsdR<sup>-</sup> res<sup>-</sup> mod<sup>+</sup> [RP4-2- Tc::Mu-Km::Tn7] (Simon, et al., Plasmid vectors for the genetic analysis and manipulation of rhizobia and other gram-negative bacteria, p. 640-659. In A. Weissbach, and H. Weissbach (eds.), Methods in enzymology, vol 118, Academic Press, Inc., Orlando, 1986).

Pseudomonas minimal salts medium (PMS) was prepared according to Giurard and Snell (Biochemical factors in growth, p. 79-111. In P. Gerhardt, R. G. E. Murray, R. N. Costilow, E. W. Nester, W. A. Wood, N. R. Krieg, and G. B. Phillips (eds.), Manual of methods for general bacteriology, American Society for Microbiology, Washington, DC., 1981) and contained 0.2% glycerol, 40 mM phosphate buffer (pH 6.8), 2% Hutner's mineral base, and 0.1% (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>. PMS medium lacking sulfate was prepared with chloride salts in place of sulfate salts. Luria broth (LB) was 1% bactotryptone, 0.5% yeast extract, and 1% NaCl. All liquid medium incubations were performed with shaking in water baths (New Brunswick Scientific, Edison, NJ). Ampicillin (50 µg/ml) and tetracycline (12.5 µg/ml) were included as selective agents when required. Dibenzothio-  
phene (DBT) was purchased from Fluka Chemical Corporation of Ronkonkoma, NY. DBT-sulfoxide was from ICN Biochemicals of Irvine, CA, and DBT-sulfone was obtained from Aldrich Chemical Company of Milwaukee, WI. Agarose was obtained from BRL.

-32-

Plasmid vectors

pLAFR5 (Keen, et al., Gene 70:191-197, 1988) and pRF29 (Desomer, et al., 1988) served as sources of the Rhodococcus plasmid origin of replication.

5 Cosmid library construction

High molecular weight DNA was isolated from IGTS8 by the method of Consevage et al., (J. Bacteriol., 162:138-146, 1985), except that cell lysis was accomplished in TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) containing lysozyme (5 mg/ml) and SDS (2%). The DNA was partially digested with Sau3AI and fragments of approximately 20 kb were isolated after centrifugation through a sodium chloride gradient (Frischauf, et al., Digestion of DNA: size fractionation, p. 183-189. In S. L. Berge, and A. R. Kimmel (eds.), 15 Methods in Enzymology, vol 152, Academic Press, Inc, San Diego, CA, 1987). These fragments were ligated into the BamHI site of pLAFR5 using standard procedures. In vitro packaging was performed using Gigapack Plus (Stratagene). Packaged cosmids were transduced into E. coli S17-1.

20 DBT spray plate assay

A spray plate assay for the identification of bacteria capable of modifying dibenzothiophene (DBT) was originally described by Kiyohara et al., (Appl. Environ. Microbiol., 43:454-457, 1982) and modified by Krawiec 25 (Bacterial desulfurization of thiophenes: screening techniques and some speculations regarding the biochemical and genetic bases, p. 103-114. In G. E. Pierce (ed.), Developments in Industrial Microbiology, vol 31, Society for Industrial Microbiology, Columbus, Ohio, 1990). The assay 30 was further modified for use with R. rhodochrous IGTS8 as follows. Cells from individual IGTS8 colonies were transferred to LB plates as small (0.5 cm) patches and were incubated at 30°C for 24 to 36 h. Large amounts of cells from these patches were transferred onto PMS-1% agarose

-33-

plates that lacked a source of sulfur. These plates were immediately sprayed with a 0.1% DBT solution in ethyl ether. The PMS-DBT plates were incubated at 30°C for a minimum of 18 hours and fluorescent products around the patches were detected by viewing under short-wave (254 nm) UV illumination.

#### Sulfur bioavailability assay

IGTS8 was incubated in PMS medium at 30°C for 24 to 48 h, the cells were pelleted by centrifugation, followed by two washes with sulfur-free PMS. Washed cells were inoculated into PMS that contained, as a sole source of sulfur, a 0.2% concentration of one of the following: DBT, DBT-sulfoxide, or DBT-sulfone. The inoculum was adjusted so that the beginning absorbance at 600 nm ( $A_{600}$ ) was 0.02. The culture was incubated at 30°C and growth was monitored at  $A_{600}$ . For cultures incubated with DBT, the supernatant was viewed at various intervals under short wave UV light to check for production of fluorescent products.

#### Plasmid isolation and hybridizations

Cosmid DNA (pLAFR5) was isolated from E. coli as described by Ish-Horowicz and Burke (Nucl. Acids Res., 9:2989-2998, 1981), and from Rhodococcus species as described by Singer and Finnerty (J. Bacteriol., 170:638-645, 1988). Large scale cosmid preparations were carried out according to Birnboim and Doly (Nucl. Acids Res., 7:1413-1423, 1979). DNA hybridization experiments were performed according to Southern (J. Molec. Biol., 98:503-517, 1975). DNA was labelled with  $^{32}\text{P}$ -dCTP (Amersham), using the random primer method of Feinberg and Vogelstein (Anal. Biochem., 137:266-267, 1984).

#### UV mutagenesis of IGTS8

IGTS8 was incubated overnight in LB at 30°C and approximately 3000 colony forming units were spread onto

-34-

fresh LB plates. These plates were immediately exposed to short wave UV light (254 nm) for 5 to 20 s at a distance of 3.5 cm. Plates were incubated at 30°C for 48 h or until colonies developed. Colonies from plates exhibiting  
5 >50% cell death were assayed for their ability to metabolize or desulfurize DBT, using the spray plate assay.

#### Electrotransformation of Rhodococcus

R. rhodochrous IGTS8 and the UV1 mutant were transformed with plasmid DNA via electroporation (Gene Pulser,  
10 Biorad Laboratories, Inc, Hercules, CA). The bacteria were grown in LB for 24 to 48 h at 30°C, diluted to an  $A_{600}$  of 0.15 with fresh LB, and incubated at 30°C for an additional 4 h. Cells were collected by centrifugation and washed four to five times with 0.3 M sucrose and finally  
15 resuspended to  $\sim 5 \times 10^9$  cells/ml in 0.5 M sucrose. To an ice cold 0.2 cm electroporation cuvette (Biorad), was added 40  $\mu$ l of this bacterial solution. The cells were pulsed at 25  $\mu$ F and 2.5 kV with the Pulse Controller at 800 ohms and were immediately diluted with 1 ml of LB  
20 containing 0.5 M sucrose. The cells were incubated at 30°C for 1 h, plated on LB agar plates plus appropriate antibiotics, and incubated at 30°C until colonies developed. When the plasmid carried the pRF29 Rhodococcus plasmid origin of replication, colonies were visible after  
25 48 h. In the absence of the pRF29 origin, colonies appeared after 4 to 5 days.

R. fascians D188-5 was transformed by electroporation in a similar manner but, due to its slower growth rate, it was incubated in LB overnight until it reached an  $A_{600}$  of  
30  $\sim 2.0$ . The cells were washed and resuspended in distilled water instead of sucrose. The Pulse Controller was set at 400 ohms and the recovery period after electroporation was in LB for 4 h before plating onto selective media. Successful transformation of R. fascians D188-5 with E. coli  
35 plasmids required that the DNA be methylated in vitro

-35-

beforehand, using the CpG methylase, SssI (New England Biolabs, Inc., Beverly, MA).

Gas chromatography and mass spectroscopy

Cells were incubated overnight in LB medium at 30°C and 100 µl was used to inoculate 50 ml of PMS minimal medium. The culture was incubated at 30°C for 4 days, washed twice with sulfur-free PMS and the pelleted cells were inoculated into 50 ml of PMS that contained 0.1% DBT as the sole source of sulfur. These cells were incubated at 30°C for 24 h and the supernatant was stored frozen at -20°C. For assays involving R. fascians D188-5, incubation times were increased 2 to 3-fold.

Sample preparation and chemical analyses were performed as described (Olson, et al., Energy & Fuels, submitted, 1993). Briefly, each sample supernatant (~50 ml) was thawed and residual insoluble material was removed by centrifugation. The cleared supernatant was acidified with HCl to pH 1.0 and then extracted three times with 50 ml of ethyl acetate. Insoluble material from the centrifugation step was also extracted with ethyl acetate. The ethyl acetate extracts were combined, dried over anhydrous calcium chloride, filtered, and ethyl acetate was removed by rotary evaporation. A known amount of internal standard (octadecane in chloroform solution) was added to the sample, which was then analyzed by GC/FID (gas chromatography/flame ionization detection) and GC/FTIR/MS (gas chromatography/Fourier transform infrared/mass spectrometry). In some samples, the acidic components in the ethyl acetate extract or in the post-extraction aqueous layer were methylated by treating with an ether solution of diazomethane.

The analyses were performed on a serially interfaced GC/FTIR/MS system as previously described (Diehl, et al., Spectros. Int. J., 8:43-72, 1990, Olson and Diehl, Anal. Chem., 59:443-448, 1987). This system consisted of the

-36-

Finnegan ion trap (ITD 800) operated with the AGC on and the Nicolet 20SXB Fourier transform infrared spectrometer. Gas chromatography was conducted with a 30 m x 0.32 mm DB5 column (1.0  $\mu$ m phase thickness) with a 2.0 ml/min helium carrier flow rate measured at 330°C. On-column injections were utilized for sample introduction because the sulf-oxides and sulfones are thermally unstable and they decompose in split or splitless injectors (Vignier, et al., J. High Resol. Chromatogr. & Chromatogr. Commun., 6:661-665, 1983). The oven temperature program was as follows: 40°C injection, followed by increases in temperature at rates of 20°C/min to 80°C, 5°C/min to 200°C, 10°C/min to 330°C, and hold for 5 min. GC/FID analyses were performed with a HP 5880A with a similar column and program for flow rate and oven temperature.

## Results

### Isolation of a Dsz- Mutant of R. rhodochrous IGTS8

When cloning from a foreign bacterial genus into E. coli, not all genes are expressed nor are all protein products active. To assure that cloned desulfurization genes would be expressed in the host cell, a mutant of R. rhodochrous IGTS8 that could no longer desulfurize DBT was isolated. Using this mutant as a cloning recipient would insure that the cellular environment was appropriate for gene expression and protein function, thereby allowing screening for cloned desulfurization genes by complementation.

R. rhodochrous IGTS8 was mutagenized by exposure to UV light, and 1000 survivors were screened for the ability to produce a UV fluorescent product in the DBT spray plate assay. Three potential desulfurization negative mutants were identified and then re-evaluated in the sulfur bio-availability assay. Two mutants (designated UV1 and UV23) could not use DBT or DBT-sulfone as sole sources of sulfur and thus appeared to be Dsz-. When gr wn in the presence

-37-

of DBT, mutant UV1 could not metabolize DBT to 2-HBP or to any other potential intermediate, as measured by GC/MS analysis. Therefore, strain UV1 was considered to be Dsz- and was used as the host for complementation studies to  
5 identify clones that carried desulfurization genes.

#### Cosmid cloning of desulfurization genes

DNA from Dsz+ source organism IGTS8 was used to construct a library in the cosmid vector, pLAFR5. This library was transduced into E. coli S17-1 and plasmids  
10 were isolated from approximately 25,000 colonies. These cosmids were electroporated into R. rhodochrous UV1, a Dsz- mutant of IGTS8, with an efficiency of ~300 transformants/ $\mu$ g DNA. Various numbers of UV1 transformants were pooled and incubated for 18 hours at 30°C, after  
15 which the cells were washed twice and resuspended in sulfate-free PMS. Approximately  $7 \times 10^8$  pooled cells were inoculated into 100 ml of PMS with DBT as the sole source of sulfur. A predicted product of the DBT desulfurization reaction is 2-HBP, which is fluorescent when exposed to UV  
20 light. Therefore, batch cultures were grown at 30°C and the supernatants were observed for fluorescence. Approximately 3300 UV1 transformants were screened in four separate batches. In one batch (representing ~600 transformants) a UV fluorescent product appeared in the supernatant  
25 after five days' incubation. Individual colonies were isolated and twelve of these continued to produce a fluorescent product when exposed to DBT.

Attempts to recover cosmid DNA from these isolates failed, so Southern hybridizations were performed to  
30 determine if the cosmids had become integrated into the chromosome of strain UV1. Chromosomal DNA was isolated from seven transformants and digested with EcoRI. After agarose electrophoresis and blotting, the fragments were hybridized with  $^{32}$ P-labelled probes derived from pLAFR5.  
35 In all transformants tested, pLAFR5 probes hybridized to a

-38-

DNA fragment ~20 kb in size. Vector derived probes did not hybridize to the control IGTS8 genome. Therefore, the desulfurization positive cosmid clones had apparently integrated into the chromosome of strain UV1.

5        Since the plasmids had integrated into the chromosome, the genomic DNA connected to either side of the plasmid cloning site must represent R. rhodochrous IGTS8 sequences that were able to complement the Dsz- mutation in strain UV1. (This would be true regardless of whether  
10 the mode of integration was by homologous or illegitimate recombination.) Sequences were recovered that flanked the inserted plasmid from three desulfurization positive transformants by digesting genomic preparations with EcoRI or BamHI. These enzymes cut pLAFR5 once in the polylinker  
15 region so that an intact sequence of pLAFR5 could be recovered, linked to a neighboring chromosomal fragment from IGTS8. The digested DNA was ligated to itself (at a concentration of ~20 ng/ $\mu$ l) and was transformed into E. coli S17-1. Sixteen tetracycline resistant colonies were  
20 obtained, seven from the BamHI digestion and nine from the EcoRI digestion. Restriction enzyme analysis revealed that all the EcoRI-rescued clones contained a 2.1 kb fragment of IGTS8 DNA. The BamHI-rescued clones contained a 1.65 kb fragment from IGTS8.

25        The 2.1 kb IGTS8 DNA from the EcoRI rescue experiment was used as a template to make labelled DNA probes, which were hybridized to colony lifts of the original, intact cosmid library in E. coli. Of 5000 colonies, 17 hybridized with the IGTS8 probes. Cosmid DNA was isolated from  
30 each clone and transformed into strain UV1. Three of the seventeen DNA preparations complemented the Dsz- phenotype.

      A restriction map for this region was constructed, using EcoRI and HindIII. Probes from the 2.1 kb IGTS8 DNA  
35 hybridized to the 4.5 kb EcoRI fragment. All cosmid clones that conferred the Dsz+ phenotype contained the



-39-

entire 4.5 kb EcoRI fragment and portions of the 4.5 kb EcoRI-HindIII and 18 kb EcoRI fragments. These results indicated that the desulfurization genes lay within a 15 kb region.

#### 5 Subcloning the desulfurization genes

The 4.5 kb EcoRI and the 4.5 kb EcoRI-HindIII fragments were subcloned into pLAFR5, but neither fragment complemented the Dsz<sup>-</sup> mutation of strain UV1. The 9.0 kb EcoRI fragment from GE1-H, the 15.0 kb EcoRI-HindIII fragment from GE1-C, and the 18 kb EcoRI fragment from GE1-K were subcloned into pLAFR5 to yield the plasmids pSAD60-28, pSAD48-12, and pSAD56-6, respectively. When transformed into UV1, all three produced UV fluorescent products from DBT in the spray plate assay, consistent with the localization of the Dsz<sup>+</sup> phenotype as determined by restriction mapping. Construction of additional subclones from this region narrowed the location of the relevant genes to a 6.5 kb BstBI fragment.

#### 20 Nature of the mutation in strain UV1

Genomic blots of EcoRI digested IGTS8 and UV1 DNA were hybridized with probes produced from the 2.1 kb EcoRI-rescued fragment of IGTS8. No hybridization was detected to UV1 DNA, indicating that the UV1 mutation is a large deletion and not a simple point mutation.

#### 25 A Rhodococcus plasmid origin of replication increases transformation of UV1

Electroporation of UV1 with pSAD48-12 typically resulted in a low transformation efficiency (~550/μg DNA) and only about 50% of the transformants exhibited the Dsz<sup>+</sup> phenotype (presumably because DNA had been lost or rearranged during recombination with the chromosome). To improve the transformation efficiency, a 4.5 kb HindIII fragment from pRF29 was cloned into the HindIII site of

-40-

pSAD48-12, resulting in pSAD74-12. This 4.5 kb fragment contains a Rhodococcus plasmid origin of replication, which allowed pSAD74-12 to replicate as a plasmid in strain UV1. This clone transformed UV1 with an efficiency of greater than  $10^4$  transformants/ $\mu$ g DNA. Nearly 100% of these transformants exhibited the Dsz+ phenotype. Unfortunately, the yield of plasmid prepared directly from UV1 was so poor that DNA from minipreparations could not be visualized on agarose gels. However, plasmid isolated from UV1 could be used to transform E. coli S17-1, from which large amounts of the plasmid were prepared.

The Dsz+ phenotype is not expressed in E. coli S17-1

E. coli S17-1 was transformed with pSAD48-12 and desulfurization activity was measured with the spray plate assay. No positive colonies were identified. It was possible that the E. coli polymerase could not recognize the IGTS8 promoter(s) in pSAD48-12, so the IGTS8 DNA was placed under control of the E. coli lac promoter. The 15 kb EcoRI-HindIII IGTS8 fragment from pSAD48-12 was subcloned into the pBluescript vectors, SK' and KS', so that the IGTS8 fragment was cloned in both orientations with respect to the lac promoter. Neither clone expressed the Dsz+ phenotype in E. coli XL1-Blue. It is not yet known whether this stems from poor transcription or translation of the cloned genes or whether the overproduced proteins are inactive in E. coli S17-1.

The Dsz+ gene or genes are expressed in R. fascians

Since the cloned genes were either not expressed or produced inactive proteins in E. coli, efforts were initiated to express the genes in other Rhodococcus species. R. fascians D188-5 exhibited no desulfurization in the DBT spray plate assay or in the sulfur bioavailability assay. Initial attempts to transform R. fascians with the desulfurization positive plasmid, pSAD74-12 were unsuccessful.

-41-

Other Rhodococcus species are known to have endogenous restriction systems that cleave DNA at SalI-like restriction sites. Since pSAD74-12 contained multiple SalI recognition sequences, CpG methylase, SssI, was used to methylate pSAD74-12 in vitro. With methylated pSAD74-12 DNA, transformants of R. fascians D188-5 were obtained with an efficiency of about  $7 \times 10^3$  transformants/ $\mu$ g DNA. These transformants displayed the Dsz+ phenotype in the spray plate assay and GC analysis of liquid medium supernatant revealed the formation of 2-HBP from DBT.

Efforts to transform pSAD74-12 into a second species, R. rhodochrous ATCC13808 were ineffective, despite the use of unmethylated or CpG-methylated plasmid. It is possible that the electroporation conditions for ATCC13808 were not optimal, though a wide range of conditions was tested. It seems more likely that ATCC13808 has a restriction system that is not inhibited by CpG methylation.

#### 2-HBP is the major desulfurization product

The predominant metabolite produced from DBT by R. rhodochrous IGTS8 is 2-HBP, with small amounts of 2'-hydroxybiphenyl-2-sulfinic acid (DBT-sultine) and 2'-hydroxybiphenyl-2-sulfonic acid (DBT-sultone) also identified by GC/MS analysis (Olson, et al., Energy & Fuels in press, 1993). These products were also produced by IGTS8 in this work (Table 2). Neither R. fascians D188-5 nor R. rhodochrous Dsz- mutant UV1 produced these products from DBT. However, when R. fascians D188-5 was transformed with plasmid pSAD74-12 and when the R. rhodochrous UV1 mutant was transformed with plasmid pSAD104-10, these bacteria produced products from DBT that were identical to those identified for R. rhodochrous IGTS8 (Table 2). In particular, 2-HBP was produced in large quantities, indicating that carbon-sulfur bond specific desulfurization of DBT was mediated by products of genes cloned from IGTS8.

-42-

One subclone, pSAD90-11, carried a DNA fragment that was supposedly identical to that cloned into pSAD104-10, but the two plasmids differed in the results they produced when introduced into R. rhodococcus UV1. In the plate assay, the surface film of DBT disappeared from the vicinity of colonies that contained pSAD104-10, producing a clear zone, and a fluorescent halo appeared around those colonies. On the other hand, when cells contained pSAD90-11, no fluorescent products were produced but a zone of DBT clearing did form around each colony. GC/MS analysis showed that no 2-HBP was produced by cells containing pSAD90-11, but that a significant amount of DBT-sultone did accumulate (Table 2). The sultone does not accumulate in the parent strain, UV1 (data not shown). These observations imply that when the 9.0 kb EcoRI fragment was subcloned into pSAD90-11 the DNA was damaged so as to inactivate the gene(s) encoding the enzyme(s) that convert the sultone to 2-HBP. This suggests that at least two enzymes are involved in desulfurization and that the sultone may be an intermediate in the pathway. This result is consistent with the kinds of metabolites detected in the original isolate, R. rhodochrous IGTS8 (Olson, et al., 1993).

-43-

Table 2  
Metabolites produced from DBT by Rhodococcus species transformed with subclones derived from R. rhodochrous IGTS8.

Metabolite <sup>a</sup>	<u>Rhodococcus</u> species (plasmid)			
	<u>R. rhodochrous</u> IGTS8	UV1 (psAD104-10) <sup>b</sup>	UV1 (psAD90-11) <sup>c</sup>	<u>R. fascians</u> D188-5 (psAD-74-12) <sup>d</sup>
DBT	++++ <sup>e</sup>	++++ <sup>e</sup>	++++	++
DBTO	+	0	0	0
DBTO <sub>2</sub>	0	0	0	0
DBT-sultone	+	++	++	+
DBT-sultine	0 or trace	0	trace	+
2-HBP	++++	++++	0	+++

a Products are: DBT, dibenzothiophene; DBTO, dibenzothiophene 5-oxide (sulfoxide); DBTO<sub>2</sub>, dibenzothiophene 5,5-dioxide (sulfone); DBT-sultone, 2'-hydroxybi-

5

10

-44-

phenyl-2-sulfonic acid (detected as dibenz[c,e][1,2]-oxathiin 6,6-dioxide); DBT-sultine, 2'-hydroxybiphenyl-2-sulfinic acid (detected as dibenz[c,e][1,2]-oxathiin 6-oxide); dibenzothiophene sulfone; 2-HBP, 2-hydroxybiphenyl (Krawiec, pg. 103-114. In G. E. Pierce (ed.), Developments in Industrial Microbiology, vol 31, Society for Industrial Microbiology, Columbus, Ohio, 1990).

- b 9.0 kb EcoRI DNA fragment from IGTS8 subcloned into pLAFR5, plus the origin of replication from pRF29.
- c Mutated 9.0 kb EcoRI DNA fragment from IGTS8 subcloned into pLAFR5, plus the origin of replication from pRF29.
- d 15.0 kb EcoRI-HindIII DNA fragment from IGTS8 subcloned into pLAFR5, plus the origin of replication from pRF29.
- e Presence of metabolites is reported in relative amounts from very large amounts (+++++) to very small (+), i.e., trace amounts.

20 IGTS8 cannot use DBT-sulfoxide as a sulfur source

R. rhodochrous IGTS8 was incubated in minimal medium with one of the following as the sole source of sulfur:

-45-

DBT, DBT-sulfoxide, or DBT-sulfone. IGTS8 was incapable of utilizing the sulfur supplied by DBT-sulfoxide but grew well in the presence of DBT or DBT-sulfone. DBT-sulfoxide was not toxic to cells when grown in a rich medium (LB).

- 5 Therefore, either IGTS8 cannot transport or otherwise act on DBT-sulfoxide, or else DBT-sulfoxide is not a true intermediate of the desulfurization pathway.

EXAMPLE 4: DNA SEQUENCING OF A 9763 NUCLEOTIDE ECORI-SAU3AI FRAGMENT CONTAINING THE GENE OR GENES FOR THE DESULFURIZATION BIOCATALYST OF IGTS8 BY THE METHOD OF SANGER ET AL.

10

A 9763 nucleotide EcoRI-Sau3AI fragment containing the gene or genes responsible for the Dsz<sup>+</sup> phenotype was isolated from the IGTS8 source organism. The DNA sequence of this fragment was determined from both strands of DNA using the dideoxy chain-termination method of Sanger et al. (1977), DNA sequencing with chain-termination inhibitors, 74 PROC. NATL. ACAD. SCI. USA 5463-5467, a modified T7 DNA polymerase (USB) and [ $\alpha$ -<sup>35</sup>S]-dCTP (Amersham). Deletion clones for DNA sequencing were constructed in pBluescript (Stratagene) using exonuclease III and the methods of Henikoff (1984), Unidirectional digestion with exonuclease III creates targeted breakpoints for DNA sequencing, 28 GENE 351-359.

15

20

- 25 Sequences from 141 individual deletion clones were used to reconstruct the entire 9763 nucleotide fragment.

-46-

Computerized sequence assembly was performed using DNA InspectorII (Textco, Hanover, NH). The DNA sequence was determined independently for each strand of DNA, but the entire 9763 nucleotide fragment was not completely sequenced on both strands. The sequence determined from one strand of DNA covered 95% of the 9763 nucleotide sequence. On the other DNA strand, 96% of the sequence was determined. The sequence was determined from at least two independent deletion clones for the entire 9763 nucleotide fragment.

EXAMPLE 5: FURTHER RESOLUTION OF THE SEQUENCE OF PTOXI-1 AND OPEN READING FRAMES (ORFS) ENCODED THEREIN; DSZ+ PROMOTER ENGINEERING; EXPRESSION OF THE DSZ+ PHENOTYPE IN A HETEROLOGOUS HOST ORGANISM; MAXICELL ANALYSIS OF DESULFURIZATION GENE EXPRESSION PRODUCTS

Organization of the desulfurization cluster

Sequencing of pTOXI-1, the results of which are set forth below in the Sequence Listing, predicted three nearly contiguous open reading frames (ORFs) on one strand of the clone (Figure 7). The sizes of each ORF are predicated as 1359 bases (bps 786-2144) for ORF 1, 1095 bases (bps 2144-3238) for ORF 2 and 1251 bases (bps 3252-4502) for ORF 3. Subclone analysis described below has revealed that ORFs 1, 2 and 3 are required for the conversion of DBT to 2-HBP and that all of the genes encoded by these ORFs are transcribed



-47-

on a single transcript as an operon. All subclones described below are maintained in E. coli - Rhodococcus shuttle vector pRR-6. Activity of each subclone was determined by growing transformants of Dsz- strain CPE-648 in a rich media (RM) for 48 hours. 1 ml of the culture was used to inoculate 25 ml BSM supplemented with greater than 100  $\mu$ M DBT or DBT-sulfone. Cultures were assayed for desulfurization products after 48 - 120 hours. A diagram of each of the subcloned fragments is shown in Figure 8.

10 In subsequent studies, the subclones were grown in rich media with chloramphenicol, then crossed into BSM supplemented with 100  $\mu$ M of either DBT or DBT-sulfone. Cultures were shaken at 30°C for 2-5 days and assayed for desulfurization products by HPLC.

15 A. pENOK-1: A subclone was constructed which contains the 4.0 kb SphI fragment of pTOXI-1. This fragment spans ORFs 1 and 2 but truncates ORF 3. Analysis of pENOK-1 containing transformants revealed the production of no products when incubated with DBT. However these transformants were capable of producing 2-HBP from DBT-sulfone.

20 B. pENOK-2: A subclone which contains the 3.6 kb SacI fragment of pTOXI-1 was constructed. This fragment contains ORFs 2 and 3 but truncates ORF 1. Analysis of pENOK-2 transformants revealed no production of any desulfurization products from either DBT or DBT-sulfone. The lack

25

-48-

of any activity detectable from either ORFs 2 or 3 suggests that the ORFs are arranged as an operon with transcription mediated from a single upstream promoter. Presumably, this promoter has been removed in this subclone.

- 5 C. pENOK-3: A 1.1 kb XhoI deletion mutation of pTOXI-1 was constructed. Both ORFs 1 and 2 are truncated. ORF 3 remains intact. Transformants harboring pENOK-3 show production of DBT-sulfone from DBT. No production of 2-HBP is detected from either DBT or DBT-sulfone. It should also  
10 be noted that at the nucleotide level, a deletion of this type would not result in a polar mutation. The sequence predicts an in-frame splicing of ORFs 1 and 2 which would produce a hybrid protein that is presumably inactive. However, by avoiding stop codons, the putative single mRNA  
15 transcript remains protected by ribosomes allowing for translation of ORF 3. The ability of the ORF-3 product to produce DBT-sulfone from DBT demonstrates that DBT-sulfone is a true intermediate in the carbon-sulfur bond specific biocatalytic desulfurization pathway of IGTS8.
- 20 D. pENOK-11: The 3.4 kb NcoI fragment from pTOXI-1 was subcloned into a unique NcoI site of pRR-6. This fragment contains all of ORFs 2 and 3 but truncates the 5' end of ORF1. Transformants with pENOK-11 demonstrated no desulfu-  
25 sulfone. This indicates essential coding regions bordering

-49-

this fragment. This is consistent with the predication that the entire cluster is expressed on a single transcript as discussed for subclone pENOK-2. Again, the promoter for gene transcription is not present in this subclone. Sub-  
5 clone pENOK-13 (below) corroborates this prediction.

E. pENOK-13: A subclone of pTOXI-1 was constructed which had a 2.6 kb SphI-XhoI deletion. This subclone only contains an intact ORF 3. ORF 1 is lost completely and ORF 2 is truncated. This subclone showed no desulfurizing-specific enzymatic activity towards DBT or DBT-sulfone. This  
10 result should be compared with the phenotype of pENOK-3 which demonstrated production of DBT-sulfone from DBT. Because pENOK-13 differs from pENOK-3 by the additional deletion of the smaller SphI/XhoI fragment, this would  
15 indicate an element in the 1.6 kb SphI/XhoI fragment which is essential for gene expression. Because sequencing has revealed no significant ORF's contained in this region, it is postulated that a promoter element may be present in this region.

20 F. pENOK-16: A subclone of pTOXI-1 was designed which eliminates nearly all unnecessary sequences from the desulfurization cluster. This construct contains the 4 kb BstBI-SnaBI which presumably contains all essential sequence for complete desulfurization in that it contains all  
25 of ORFs 1, 2 and 3 as well as 234 bases of upstream se-

-50-

quence. The 3' SnaBI site lies 80 base pairs beyond the termination of ORF 3. CPE-648 harboring this plasmid was capable of converting DBT and DBT-sulfone to 2-HBP. pENOK-16 thus represents the smallest amount of the cluster yet  
5 observed which demonstrates the complete desulfurization phenotype.

G. pENOK-18: This subclone contains a NsiI-BfaI fragment of pTOXI-1. The NsiI site is 23 bp downstream of the predicted start site of ORF 1. CPE-648 harboring this  
10 subclone lacks desulfurization activity on both DBT and DBT-sulfone. This subclone most likely eliminates the promoter region and truncates the first structural gene.

H. pENOK-Nsi: To help further elucidate the start site of ORF 1, a subclone was made in which a 4 bp deletion is  
15 introduced at the unique NsiI site which is 23 bp downstream of the predicted start site of ORF1. The mutation was generated by cutting with NsiI and blunting the ends with T4 DNA Polymerase. If the NsiI site is within the first structural gene this frameshift mutation would cause  
20 an early stop signal in ORF 1. Transformants of pENOK-Nsi were capable of producing DBT-sulfone from DBT. However, no production of 2-HBP was detected indicating that the mutation had disrupted an essential structural gene.

In subsequent studies, due to the clear expression of  
25 the ORF-3 encoded oxidase, in this clone, it was considered

-51-

likely that the ORF-2 product would also be expressed. Accordingly, ORF-2 alone is incapable of further metabolism of DBT-sulfone.

I. pENOK-19: A subclone of pTOXI-1 was constructed which  
5 contains a deletion from the NotI site, which is in the earlier part of ORF 2, to the SnaBI which is after ORF 3. This subclone should demonstrate the activity of ORF 1 alone. CPE648 transformants harboring this subclone displayed no enzymatic activity towards DBT or DBT-sulfone.  
10 The results of pENOK-Nsi and pENOK-19, taken together, suggest that the ORF-I and ORF-2 products must be simultaneously expressed in order to further metabolize DBT-sulfone.

J. pENOK-20: In order to evaluate the function of ORFs 2  
15 and 3 separately from ORF 1, DNA spanning ORFs 2 and 3 was amplified by the Polymerase Chain Reaction (PCR). Primers RAP-1 (5'-GCGAATTCGACCGAGTACC-3', bps 2062-2082) and RAP-2 (5'-ATCCATATGCGCACTACGAATCC-3' bps 4908-4886) were synthesized with the Applied Biosystems 392 DNA/RNA Synthes-  
20 izer. Nucleotides in bold were altered from the template sequence in order to create restriction sites for subcloning; thus primer RAP-1 contains an EcoRI site, and primer RAP-2 contains an NdeI site. Amplification was carried out with the GeneAmp Kit (Perkin Elmer Cetus) which utilizes

-52-

the Taq polymerase and the Perkin Elmer Cetus 9600 Thermocycler. Parameters were as follows:

Template:	pMELV-1 Plasmid DNA	0.2 or 2.0 ng
Primers:	RAP-1	0.5 or 0.2 $\mu$ M
5	RAP-2	0.5 or 0.2 $\mu$ M
Cycles:	1X@	96°C 2 min
	25X@	96°C 30 sec
		52°C 30 sec
		72°C 2 min

10 Amplification yielded the predicted 2846 bp fragment. In order to express the amplified fragment harboring ORFs 2 and 3, it was ligated to the XbaI/EcoRI fragment of the chloramphenicol resistance gene promoter from Rhodococcus fascians (Desomer et al.: Molecular Microbiology (1992) 6  
15 (16), 2377-2385) to give plasmid pOTTO-1. Ultimately, a blunt end ligation was used for the subcloning of the amplified product due to the fact that ligation using the engineered restriction sites was unsuccessful. This fusion was ligated to shuttle-vector pRR-6 to produce plasmid  
20 pENOK-20. CPE648 transformants of pENOK-20 were grown in the presence of DBT and 25  $\mu$ g/ml chloramphenicol for promoter induction. All transformants converted DBT to DBT-sulfone presumably through the activity of the ORF 3 as demonstrated in subclone pENOK-3. The inability to further  
25 process DBT-sulfone with the presence of ORF 2 suggests that the product of ORF 2 alone is incapable of using DBT-sulfone as a substrate. This is consistent with results

-53-

obtained from pENOK-Nsi, and suggests that ORF-2 alone is incapable of using DBT-sulfone as a substrate.

Assignment of Gene Products of ORFs 1, 2 and 3

Based on the foregoing subclone analyses, functions  
5 have been tentatively assigned to each of the ORFs present within the pTOXI-1 sequence. ORF 3 can be identified as responsible for an oxidase capable of conversion of DBT to DBT-sulfone. Subclone pENOK-3 demonstrates this activity very clearly. ORFs 1 and 2 appear to be responsible for  
10 conversion of DBT-sulfone to 2-HBP. This aryl sulfatase activity is evidenced in subclone pENOK-1. However subclones pENOK-19 and pENOK-20 indicate that neither ORF 1 or ORF 2 alone is capable of any conversion of the intermediate DBT-sulfone. This suggests that the protein products of ORFs 1 and 2 work together to cleave both of the  
15 carbon-sulfur bonds. Presumably, this is achieved through a heterodimer arrangement of the proteins, or through a regulatory function of one protein on the other. The results of parallel investigations, presented in Example 3,  
20 suggested that ORF-1 encodes an enzyme that converts DBT-sulfone to DBT-sultone. Lengthy incubations of CPE-648 harboring pENOK-19 (intact native promoter and ORF-I) have shown neither the depletion of DBT-sulfone nor the production of any new products. This is contrary to indications derived from Example 3.  
25

-54-

Alternative Promoter Screening

Increasing the specific activity of desulfurization is a significant objective of the studies described herein. One approach to accomplishing this goal is to replace the original promoter with one that can produce both higher and constitutive expression of the desulfurization gene cluster. Because there are so few reported and characterized Rhodococcus promoters, random genomic libraries have been prepared and screened for promoter activity in two systems. In one, the reporter is the chloramphenicol resistance gene used in the above-discussed plasmid constructions. In the other, the desulfurization cluster itself is used as a reporter.

Promoter Screening Example A. Chloramphenicol Resistance Reporter.

As also described below, partially digested Rhodococcus genomic DNA has been cloned upstream of a promoterless chloramphenicol resistance gene. The resulting libraries were then transformed into Rhodococcus which are subjected to chloramphenicol selection. Four apparent promoter elements were rescued by pRHODOPRO-2, although plasmid could be isolated from only one of these, possibly due to vector instability. The stable plasmid RP2-2A has been subjected to sequence analysis. Technical problems have been observed with restriction enzyme treatment of the NarI cloning site used in these vectors. Unfortunately,



-55-

the NarI enzyme demonstrates severe site-selectivity and does not appear to digest the vector well. New vectors have been constructed in order to alleviate this problem, although a lack of convenient and unique restriction sites  
5 slowed the progress of these studies. A recent observation on the Rhodococcus replication origin will aid in constructing a more effective promoter probe, as discussed below.

Recently, the 1.4kb BglII fragment was removed from  
10 pRR-6, and the ends were blunted and religated to produce pRR-12 (Figure 9), which contains no BglII sites. Desomer et al. (Molecular Microbiology (1992) 6 (16), 2377-2385) reported that this region was needed for plasmid replication. Thus, it was surprising that this construct was  
15 capable of producing Cm<sup>r</sup> transformants, indicating that this region was not essential for plasmid replication in the strain of organisms used for the present studies. This observation forms the conceptual basis for construction of a vector that will utilize a synthesized BglII site for  
20 cloning the random genomic fragments. BglII accepts DNA digested by Sau3A, an effective and frequent cutter of IGTS8 DNA. These constructs are expected to allow for the production of better, more representative random genomic libraries.

-56-

Promoter Screening Example B: Desulfurization Cluster  
Reporter.

Vector pKAMI has been used as a second direct "shot--  
gun" approach to finding a suitable alternative promoter  
5 (Figure 10). An NdeI site was engineered upstream of the  
promoterless Dsz cluster to serve as the site of insertion  
of random genomic DNA (from strains GPE-362, CPE-648 and  
IGTS8) fractionated by NdeI and the compatible 4bp cutters  
MseI and BfaI. Originally, this ligation mixture was  
10 directly transformed into GPE-362 cells, which were then  
used en masse to inoculate 250 ml BSM + DBT. These efforts  
were undertaken with the goal of amplifying a superior Dsz+  
strain due to its ability to utilize DBT as the sole source  
of sulfur. To date, 14 transformations of this type have  
15 been done. Of these, all but 2 have resulted in producing  
Dsz+ cultures. Eleven individual clones have been isolated  
and characterized. These are capable of low-level (0.6 -  
1.0 mg/L 2-HBP/OD<sub>600</sub>/hr), constitutive expression of the  
desulfurization trait. Restriction analysis of plasmids  
20 isolated from these eleven has revealed that all but one  
(KB4-3) are simple rearrangements of the pKAMI backbone  
resulting in gratuitous expression from vector borne pro-  
moters. Many of the rescued plasmids show identical re-  
striction patterns although originating from separate  
25 ligations, suggesting an inherent vector instability. It  
appears as if, with this type of selection, rearrangements

-57-

of pKAMI that utilize a vector promoter sequence are strongly selected.

The above-described selection procedure has thus given way to a promoter screen geared to minimize the plasmid rearrangement. In this procedure, the pKAMI/genomic library is first amplified in E. coli, then the individual JM109 colonies are pooled together. The plasmids are extracted, and used to transform Dsz- strain GPE-362. Instead of using en masse enrichment, the GPE362 transformations are plated to Rich Media + chloramphenicol for selection of plasmid containing cells. Resulting colonies are replica-plated to BSM agarose + DBT plates, then checked for desulfurization activity by UV fluorescence production. Over 7,000 GPE-362 transformants have been screened in this fashion. Thirty-six have been isolated from these which produce UV fluorescence on BSM + DBT plates. Current efforts focus on the identification and characterization of the engineered plasmids borne by these 36 transformants.

## 20 Alternative Promotor Engineering

The close physical arrangement of the three ORFs of pTOXI-1 does not provide sufficient space for promoters for either ORFs 2 or 3. This fact, coupled with the results of the subclone analysis in which intact ORFs 2 and 3 provided no activity (see pENOK-2, pENOK-11, and pENOK-13), suggested that this cluster of genes is organized as an operon

-58-

with only one promoter for expression of the three genes. Given that the desulfurization trait of IGTS8 is repressed by sulfate (Kilbane and Bielaga, Final Report D.O.E. Contract No. DE-AC22-88PC8891 (1991), it is possible that the  
5 operon promoter is tightly controlled by sulfur levels. With the elucidation of the molecular arrangement of the desulfurization cluster, alternative promoters can be rationally engineered to eliminate the sulfur repression, increase expression of the desulfurization genes and there-  
10 by increase the specific activity of the Dsz<sup>+</sup> trait.

Examples of potential alternative promoters include other known and described promoters such as the chloramphenicol resistance gene promoter from Rhodococcus fascians (Desomer et al.: Molecular Microbiology (1992) 6 (16),  
15 2377-2385), the nitrile hydratase gene promoter from Rhodococcus rhodochrous (Kobayashi, et al.: Biochimica et Biophysica Acta, 1129 (1991) 23-33), or other strong promoters isolated from Rhodococcus sp. by "shot-gun" promoter probing. Other potential alternative promoters include those  
20 from other Gram positive organisms such as Corynebacterium, Bacillus, Streptomyces, and the like.

Promoter Engineering Example A: Expression from the chloramphenicol resistance gene promoter from Rhodococcus fascians.

25 pSBG-2 (Figure 11). The promoterless desulfurization cluster was isolated from pTOXI-1 as a 4.0 kb DraI/SnaBI

-59-

fragment and ligated to a unique blunted AflIII site of pRR-6. This ligation inserted the cluster downstream of the chloramphenicol resistance gene promoter and upstream of the resistance structural gene. Thus, messenger RNA (mRNA) transcription should proceed through the Dsz gene cluster and proceed on to the resistance gene. However, original selections of transformants on chloramphenicol did not yield transformants, suggesting poor transcriptional read-through. Dsz+ transformants harboring the plasmid were selected first through sulfur bioavailability assays and secondarily on chloramphenicol plates. Unlike IGTS8, pSBG-2 transformants are capable of converting DBT to 2-HBP in BSM media supplemented with 20 mM Na<sub>2</sub>SO<sub>4</sub>, which demonstrates the removal of sulfate repression by promoter replacement. Specific activity of transformants was measured between 0.9 and 1.7 mg 2-HBP/l/OD<sub>600</sub>/hr for a 16 hr culture in a rich media (RM) supplemented with 25 µg/ml chloramphenicol.

pSBG-3. The Rhodococcus origin of replication was removed from pSBG-2 by elimination of the 4.0 kb XbaI fragment. Without the origin, transformation is obtainable only through integration. CPE-648 transformants with this plasmid were selected on RM + chloramphenicol and replica-plated onto BSM + DBT plates. Colonies were obtained which produced 2-HBP, as detected by fluorescence after 18 hr of incubation at 30°C.

-60-

Individual expression Of each ORF

Recently, studies have been initiated to express the three ORFs separately, each engineered with an alternative promoter. These studies are expected to elucidate the following: First, any potential rate limiting steps in the desulfurization process will be identified and overcome. Potential polarity effects of operon expression, i.e. poorer expression of downstream ORFs 2 and 3, may be causing such rate limitations. Also, given the unresolved issue of the individual functions of ORFs 1 and 2, these studies are expected to demonstrate reconstitution of DBT-sulfone to 2-HBP conversion by the separate expression of ORFs 1 and 2.

All ORFs were isolated through PCR amplification and subsequent subcloning. A typical Shine-Dalgarno sequence and a unique cloning site for alternative promoters has been engineered upstream of each ORF. Stop codons in all reading frames have been engineered downstream of each ORF to prevent read-through. Additionally, convenient flanking restriction sites for mobilization of the promoter/ORF fusions have been added to each primer. The primers used for amplification of each ORF are listed below. In-frame stop codons are marked with an asterik (\*). Sequences identical to pTOXI-I template DNA are shown in bold.

-61-

ORF1UP:

XbaI  
5'-GGAATTCTAGACATATGAGGAACAGACCATGACTCAACAACGACAAATGC-3'  
EcoRI      NdeI      Start

5 ORF1DOWN:

Stop XbaI  
3'-GTACTGTTTCGGCGCAGCTGGGGACTAAGATCTTAAGC-5'  
Stop\*      Stop      EcoRI

ORF2UP:

10 BglII  
5'-GGAATTCAGATCTCATATGAGGAAACAGACCATGACAAGCCGCGTCGACC-3'  
EcoRI      NdeI      Start

ORF2DOWN:

Stop BglII  
15 3'-CGGAGTTAGCGGTGGCTATCCTTAATCTAGACTTAAGC-5'  
Stop\*      Stop      BglII

ORF3UP:

MseI  
20 5'-GGAATTCCTTAACATATGAGGAAACAGACCATGACACTGTCACCTGA-3'  
EcoRI      NdeI      Start

ORF3DOWN:

MseI  
3'-GACTCCTAGACTCCGCGACTAATTCTTAAGC-5'  
Stop\*      Stop      Stop      EcoRI

25 Cycling parameters were: 1 x 96°C 2.0 min  
25 x 96°C 30 sec  
50°C 30 sec  
72°C 1.0 min

Each ORF has been successfully amplified and subcloned  
30 into pUC-19 NdeI as EcoRI fragments. Alternative promoters

-62-

will be ligated into the unique NdeI sites, and the fusions will be moved to Rhodococcus-E. coli shuttle vector pRR-6 for expression in Rhodococcus.

#### Heterologous Expression of the Dsz+ Trait

5 In order to determine whether plasmid pTOXI-1 contained all of the genetic material necessary for the Dsz+ trait, heterologous expression of pTOXI-1 was attempted in Rhodococcus fascians, a related organism which does not metabolize DBT (Dsz-) and in E. coli, a non-related organism which is  
10 also Dsz-.

A. Rhodococcus fascians (ATCC 12974), a Dsz- strain, was transformed with pTOXI-1. A single transformant demonstrated UV fluorescence on BSM + DBT plates, and further analysis by HPLC clearly indicated production of 2-HBP when DBT was  
15 provided as a substrate. Thus pTOXI-1 contains sufficient information to convert a heterologous Dsz- strain to the Dsz+ phenotype.

B. E. coli strain JM109 was also transformed with pTOXI-1 and was incubated with each of the substrates DBT and DBT-sulfone in either a minimal media (BSM) or a rich media (Luria Broth). In no case was production of 2-HBP observed by HPLC analysis. The inability of E. coli to express the desulfurization genes was not unexpected as gram positive genes are not universally expressible in E. coli without  
25 promoter replacement.

In order to replace the promoter of the desulfurization cluster, a 4.0 kb DraI/SnaBI fragment was isolated from pTOXI-1. This fragment contains all of the necessary structural genes but lacks the promoter sequences. This promoterless desulfurization cluster was ligated to E. coli  
30 expression vector pDR540 (Pharmacia, Piscataway, NJ) cut with BamHI and ends made blunt with Klenow. The construction fuses the tac promoter to the desulfurization cluster. The



-63-

tac promoter is under control of the lactose repressor and is repressed in a lacI<sup>q</sup> host such as JM109. Expression from the tac promoter is inducible by the addition of isopropyl  $\beta$ -D-thiogalactopyranoside (IPTG). Transformants of JM109 harboring pDRDs<sub>z</sub> grown in Luria Broth at 30°C demonstrate the Dsz<sup>+</sup> phenotype when incubated with DBT and induced with IPTG. A specific activity as high as 1.69 mg 2HBP/l/OD<sub>600</sub>/hr has been observed with pDRDs<sub>z</sub>. Activity is greatly diminished when transformants are grown at 37°C. The highest level of activity has been observed at 1hr post induction.

The above-described expression of the Dsz<sup>+</sup> trait in both a related and non-related heterologous host indicates that pTOXI-1 carries all of the genetic information required for conversion of DBT to 2-HBP.

Successful expression in E. coli provided a workable system in which the proteins encoded by the desulfurization cluster could be identified and characterized. Total protein from Dsz<sup>+</sup> cells of JM109 (pDRDs<sub>z</sub>) was isolated and examined on denaturing acrylamide gels. No novel bands could be detected with Coomassie stain. Cellular fractionation of proteins into periplasmic, cytosolic and membrane components were also analyzed by Coomassie stained gels. Again, no novel bands were detected. Without any purification, the newly expressed proteins were apparently levels too low to easily detect and resolve from background.

#### Maxicell Analysis of E. coli harboring pDRDs<sub>z</sub>

Proteins encoded by genes on plasmid DNA can be specifically radiolabeled in UV-irradiated cells of E. coli (Sancar, et al. Journal of Bacteriology. 1979, p. 692-693). This technique is known as Maxicell Analysis. Briefly, a recA strain of E. coli e.g. JM109 which harbors a plasmid is grown in M9CA medium (Maniatis et al.) to a density of  $2 \times 10^8$  cells/ml. Continuously stirred cells were then subjected to UV exposure from a Mineralight Lamp Model UVG-254 (Ultraviolet Products, Inc., San Gabriel, CA) at a distance of 10 cm

-64-

for a fluence rate of  $0.5 \text{ Joules} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ . Cells were exposed for either 60, 90 or 120 seconds. The cells were then incubated at  $37^\circ\text{C}$  for 16 hours after which they were then washed with M9 buffer and suspended in minimal medium lacking sulfate. After 1 hour of starvation at  $37^\circ\text{C}$ , [ $^{35}\text{S}$ ]methionine ( $>1000 \text{ Ci/mmol}$ ) (NEN Research Products, Boston, MA) was added at a final concentration of  $5 \mu\text{Ci/ml}$  and incubation was continued for 1 hour. Cells were collected by centrifugation and proteins isolated through a boiled cell procedure (Maniatis, et al.). Proteins were separated on an acrylamide gel. After the run, the gel was dried and subjected to autoradiography for 3 days.

Maxicells of JM109 harboring vector pDR540 showed only vector marker galactokinase protein. Maxicells of JM109 harboring vector pDRDs<sub>z</sub> showed the presence of three novel protein bands of sizes which correlated well with the predicted molecular weights of the three proteins responsible for the Ds<sub>z</sub><sup>+</sup> trait, as predicted by open reading frame analysis (see Table 3).

Table 3

Open Reading Frame	Predicted Size (kDa)	Measured Size (kDa)
ORF-1	49.5	49.5
ORF-2	38.9	33.0
ORF-3	45.1	45.0

Data obtained from Maxicell analysis thus indicated that the three predicted open reading frames of pTOXI-1 encode three structural genes which constitute the desulfurization phenotype.

The relative intensity of the three novel bands is reflective of both the number of methionine residues and the level of translation for each of the proteins. Clearly, ORF-2 with only 1 Met gives the faintest band. In

-65-

addition to the incorporation of only a single Met residue, E. coli may process the single terminal methionine, further reducing the amount of labelled protein. Therefore, the low intensity of the ORF-2 band most likely  
5 does not strictly suggest a low level of protein translation.

Interestingly, the ORF furthest from the promoter (ORF-3) appears to be present at levels comparable to ORF-1, indicating no polar effects in this operon when  
10 expressed in E. coli. It is expected that more significant information regarding protein levels will be obtained from a similar Maxicell analysis of a Rhodococcus sp. host containing plasmid pTOXI-I. Additionally, the presence of an ORF-I/ORF-2 heterodimer, postulated above,  
15 may be observable under non-denaturing conditions.

As required by 37 C.F.R. Section 1.821(f), Applicant's Attorney hereby states that the content of the "Sequence Listing" in this specification in paper form and the content of the computer-readable form (diskette)  
20 of the "Sequence Listing" are the same.

#### EQUIVALENTS

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the  
25 invention described herein. These and all other such equivalents are intended to be encompassed by the following claims.

-66-

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

## (i) APPLICANT:

(A) NAME: Energy BioSystems Corporation  
(B) STREET: 3608 Research Forest Drive B-7  
(C) CITY: The Woodlands  
(D) STATE: TX  
(E) COUNTRY: US  
(F) ZIP: 77381  
(G) TELEPHONE: 713-364-6100  
(H) TELEFAX: 713-364-6110

(ii) TITLE OF INVENTION: Recombinant DNA Encoding A  
Desulfurization Biocatalyst

(iii) NUMBER OF SEQUENCES: 5

## (iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: Hamilton, Brook, Smith and Reynolds, P.C.  
(B) STREET: Two Militia Drive  
(C) CITY: Lexington  
(D) STATE: Massachusetts  
(E) COUNTRY: U.S.A.  
(F) ZIP: 02173

## (v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk  
(B) COMPUTER: IBM PC compatible  
(C) OPERATING SYSTEM: PC-DOS/MS-DOS  
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

## (vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:  
(B) FILING DATE:  
(C) CLASSIFICATION:

## (viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: Brook, David E  
(B) REGISTRATION NUMBER: 22,592  
(C) REFERENCE/DOCKET NUMBER: EBC92-03A

## (ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: 617-861-6240  
(B) TELEFAX: 617-861-9540

## (2) INFORMATION FOR SEQ ID NO:1:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 5535 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: double  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

-67-

## (ix) FEATURE:

(A) NAME/KEY: CDS  
(B) LOCATION: 790..2151

## (ix) FEATURE:

(A) NAME/KEY: CDS  
(B) LOCATION: 3256..4506

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GCATGCACGT CGCGCCGACG CATTTCGCGC CACGGCTCCG GGCAGTTCTC GCGGCGCTGG	60
AGGCACGGAT GGGCACCCCTC AACGAACTCA CCCAAACCAC GCCGATAGCG ATCCTCGCCC	120
AAACCCTCGG CTACAGCCCT CAGACATTGG AAGCTCATGC GCGACGCATC CGGATCGACC	180
TTTGACGCT ACGTGGCGAC GCGGCTGGAC TGACGCTGGA GGTCCGACCC GACGTGTGTG	240
GTGTAGCGCC GCTTAACGGG TGCGCACGGC GGGACATCGG CCAGCTGGCT TGGCCCTCCT	300
CCGCAGGTAG TCGACCACCC CTTCCCGCAG CGGTCGGAGG TGATCGACCG TTAGGGTCAT	360
TTGCTCGCAG ATCGGCTGAT GTTGCCGATC GACGTGGTCG ACGGGACACG CTCGCGATTG	420
GCATGGCGTC CGGTGCATAC ACGACGATCT AACCAGATCG ACGGTTTTGA GCGTCGGTCA	480
ACGTCGACTC GATGCGCCGT GCGAGTGAGA TCCTTTGTGG TGCTTGGCTA TTGACCTCGA	540
CAAGGATAGA GATTGGAAG ACCTCGGATC GACCCAAATG CGGACGGCCG GCAGCGGCCA	600
AGGCGGCCAA GTCATCGGCA CCGTCACCGT CACCTTGACC CGACGTGCCC CGTGGTTCAA	660
GGCCTGAATT TGGCTGGTGG AGCATTGAAA TCAGGTGAAG TTTAACGGTG GGCACACCCC	720
GGGGGTGGGG GTGAGACTGC TTAGCGACAG GAATCTAGCC ATGATTGACA TTAAAGGAC	780
GCATACGCG ATG ACT CAA CAA CGA CAA ATG CAT CTG GCC GGT TTC TTC	828
Met Thr Gln Gln Arg Gln Met His Leu Ala Gly Phe Phe	
1 5 10	
TCG GCC GGC AAT GTG ACT CAT GCA CAT GGG GCG TGG CGG CAC ACG GAC	876
Ser Ala Gly Asn Val Thr His Ala His Gly Ala Trp Arg His Thr Asp	
15 20 25	
GCG TCG AAT GAC TTT CTG TCG GGG AAG TAC TAC CAA CAC ATC GCC CGT	924
Ala Ser Asn Asp Phe Leu Ser Gly Lys Tyr Tyr Gln His Ile Ala Arg	
30 35 40 45	
ACT CTG GAG CGC GGC AAG TTC GAT CTG TTG TTT CTG CCT GAC GGG TTG	972
Thr Leu Glu Arg Gly Lys Phe Asp Leu Leu Phe Leu Pro Asp Gly Leu	
50 55 60	
GCC GTC GAG GAC AGC TAC GGG GAC AAC CTG GAC ACC GGT GTC GGC CTG	1020
Ala Val Glu Asp Ser Tyr Gly Asp Asn Leu Asp Thr Gly Val Gly Leu	
65 70 75	
GGC GGG CAG GGT GCA GTC GCC TTG GAG CCG GCC AGT GTG GTC GCA ACC	1068
Gly Gly Gln Gly Ala Val Ala Leu Glu Pro Ala Ser Val Val Ala Thr	
80 85 90	
ATG GCC GCG GTG ACC GAG CAC CTG GGT CTT GGG GCA ACC ATT TCG GCG	1116

-68-

Met	Ala	Ala	Val	Thr	Glu	His	Leu	Gly	Leu	Gly	Ala	Thr	Ile	Ser	Ala			
95						100					105							
ACC	TAC	TAT	CCC	CCG	TAT	CAC	GTT	GCT	CGG	GTG	TTC	GCG	ACG	CTC	GAT			1164
Thr	Tyr	Tyr	Pro	Pro	Tyr	His	Val	Ala	Arg	Val	Phe	Ala	Thr	Leu	Asp			
110					115					120					125			
CAG	TTG	TCA	GGG	GGT	CGG	GTG	TCC	TGG	AAC	GTC	GTC	ACC	TCG	CTC	AAC			1212
Gln	Leu	Ser	Gly	Gly	Arg	Val	Ser	Trp	Asn	Val	Val	Thr	Ser	Leu	Asn			
				130					135					140				
GAC	GCT	GAA	GCG	CGC	AAC	TTC	GGC	ATT	AAT	CAG	CAT	CTG	GAA	CAC	GAC			1260
Asp	Ala	Glu	Ala	Arg	Asn	Phe	Gly	Ile	Asn	Gln	His	Leu	Glu	His	Asp			
			145					150					155					
GCC	CGC	TAT	GAC	CGC	GCC	GAT	GAG	TTC	TTG	GAA	GCG	GTC	AAG	AAA	CTC			1308
Ala	Arg	Tyr	Asp	Arg	Ala	Asp	Glu	Phe	Leu	Glu	Ala	Val	Lys	Lys	Leu			
		160					165					170						
TGG	AAC	AGC	TGG	GAC	GAG	GAC	GCC	CTC	GTG	CTG	GAC	AAG	GCG	GCC	GGC			1356
Trp	Asn	Ser	Trp	Asp	Glu	Asp	Ala	Leu	Val	Leu	Asp	Lys	Ala	Ala	Gly			
	175					180					185							
GTG	TTC	GCC	GAT	CCC	GCG	AAG	GTG	CAC	TAC	GTC	GAT	CAC	CAC	GGG	GAG			1404
Val	Phe	Ala	Asp	Pro	Ala	Lys	Val	His	Tyr	Val	Asp	His	His	Gly	Glu			
190					195					200				205				
TGG	CTG	AAT	GTG	CGC	GGA	CCT	CTG	CAG	GTA	CCG	CGT	TCA	CCT	CAG	GGT			1452
Trp	Leu	Asn	Val	Arg	Gly	Pro	Leu	Gln	Val	Pro	Arg	Ser	Pro	Gln	Gly			
				210					215					220				
GAG	CCG	GTG	ATC	CTG	CAG	GCC	GGC	CTG	TCG	CCC	CGG	GGT	CGG	CGC	TTC			1500
Glu	Pro	Val	Ile	Leu	Gln	Ala	Gly	Leu	Ser	Pro	Arg	Gly	Arg	Arg	Phe			
			225					230					235					
GCC	GGG	AAG	TGG	GCC	GAG	GCC	GTG	TTC	AGT	CTT	GCA	CCC	AAC	CTC	GAG			1548
Ala	Gly	Lys	Trp	Ala	Glu	Ala	Val	Phe	Ser	Leu	Ala	Pro	Asn	Leu	Glu			
		240					245					250						
GTG	ATG	CAG	GCC	ACC	TAC	CAG	GGC	ATC	AAA	GCC	GAG	GTC	GAC	GCT	GCG			1596
Val	Met	Gln	Ala	Thr	Tyr	Gln	Gly	Ile	Lys	Ala	Glu	Val	Asp	Ala	Ala			
	255					260					265							
GGG	CGC	GAT	CCC	GAT	CAG	ACG	AAA	ATC	TTC	ACC	GCC	GTG	ATG	CCG	GTA			1644
Gly	Arg	Asp	Pro	Asp	Gln	Thr	Lys	Ile	Phe	Thr	Ala	Val	Met	Pro	Val			
270					275					280				285				
CTC	GGC	GAA	AGC	CAG	GCG	GTG	GCA	CAG	GAA	CGA	CTG	GAA	TAT	CTC	AAC			1692
Leu	Gly	Glu	Ser	Gln	Ala	Val	Ala	Gln	Glu	Arg	Leu	Glu	Tyr	Leu	Asn			
				290					295					300				
AGT	CTG	GTC	CAT	CCG	GAA	GTG	GGA	CTG	TCG	ACG	CTA	TCC	AGT	CAC	ACC			1740
Ser	Leu	Val	His	Pro	Glu	Val	Gly	Leu	Ser	Thr	Leu	Ser	Ser	His	Thr			
			305					310					315					
GGC	ATC	AAC	CTG	GCG	GCG	TAC	CCT	CTC	GAC	ACT	CCG	ATC	AAG	GAC	ATC			1788
Gly	Ile	Asn	Leu	Ala	Ala	Tyr	Pro	Leu	Asp	Thr	Pro	Ile	Lys	Asp	Ile			
		320					325					330						
CTG	CGG	GAT	CTG	CAG	GAT	CGG	AAT	GTC	CCG	ACG	CAA	CTG	CAC	ATG	TTC			1836
Leu	Arg	Asp	Leu	Gln	Asp	Arg	Asn	Val	Pro	Thr	Gln	Leu	His	Met	Phe			
		335				340					345							

-69-

GCC GCC GCA ACG CAC AGC GAA GAG CTC ACG CTG GCG GAA ATG GGT CGG Ala Ala Ala Thr His Ser Glu Glu Leu Thr Leu Ala Glu Met Gly Arg 350 355 360 365	1884
CGC TAT GGA ACC AAC GTG GGG TTC GTT CCT CAG TGG GCC GGT ACC GGG Arg Tyr Gly Thr Asn Val Gly Phe Val Pro Gln Trp Ala Gly Thr Gly 370 375 380	1932
GAG CAG ATC GCT GAC GAG CTG ATC CGC CAC TTC GAG GGC GGC GCC GCG Glu Gln Ile Ala Asp Glu Leu Ile Arg His Phe Glu Gly Gly Ala Ala 385 390 395	1980
GAT GGT TTC ATC ATC TCT CCG GCC TTC CTG CCG GGC TCC TAC GAC GAG Asp Gly Phe Ile Ile Ser Pro Ala Phe Leu Pro Gly Ser Tyr Asp Glu 400 405 410	2028
TTC GTC GAC CAG GTG GTT CCG GTT CTG CAG GAT CGC GGC TAC TTC CGC Phe Val Asp Gln Val Val Pro Val Leu Gln Asp Arg Gly Tyr Phe Arg 415 420 425	2076
ACC GAG TAC CAG GGC AAC ACT CTG CGC GAC CAC TTG GGT CTG CGC GTA Thr Glu Tyr Gln Gly Asn Thr Leu Arg Asp His Leu Gly Leu Arg Val 430 435 440 445	2124
CCA CAA CTG CAA GGA CAA CCT TCA TGACAAGCCG CGTCGACCCC GCAAACCCCG Pro Gln Leu Gln Gly Gln Pro Ser 450	2178
GTTCAGAACT CGATTCCGCC ATCCGCGACA CACTGACCTA CAGCAACTGC CCGGTACCCA	2238
ACGCTCTGCT CACGGCATCG GAATCGGGCT TCCTCGACGC CGCCGGCATC GAACTCGACG	2298
TCCTCAGCGG CCAGCAGGGC ACGGTTCATT TCACCTACGA CCAGCCTGCC TACACCCGTT	2358
TTGGGGGTGA GATCCCGCCA CTGCTCAGCG AGGGGTTCG GGCACCTGGG CGCACGCGTC	2418
TACTCGGCAT CACCCCGCTC TTGGGGCGCC AGGGCTTCTT TGTCCGCGAC GACAGCCCGA	2478
TCACAGCGGC CGCCGACCTT GCCGGACGTC GAATCGGCGT CTCGGCCTCG GCAATTCGCA	2538
TCCTGCGCGG CCAGCTGGGC GACTACCTCG AGTTGGATCC CTGGCGGCAA ACGCTGGTAG	2598
CGCTGGGCTC GTGGGAGGCG CGCGCCTTGT TGCACACCCT TGAGCACGGT GAACTGGGTG	2658
TGGACGACGT CGAGCTGGTG CCGATCAGCA GTCCTGGTGT CGATGTTCCC GCTGAGCAGC	2718
TCGAAGAATC GGCGACCGTC AAGGGTGCGG ACCTCTTTCC CGATGTCGCC CGCGGTCAGG	2778
CCGCGGTGTT GGCCAGCGGA GACGTTGACG CCCTGTACAG TTGGCTGCCC TGGGCCGGGG	2838
AGTTGCAAGC CACCGGGGCC CGCCAGTGG TGGATCTCGG CCTCGATGAG CGCAATGCCT	2898
ACGCCAGTGT GTGGACGGTC AGCAGCGGGC TGGTTCGCCA GCGACCTGGC CTTGTTCAAC	2958
GA CTGGT CGA CGCGGCCGTC GACGCCGGGC TGTGGGCACG CGATCATTC GACGCGGTGA	3018
CCAGCCTGCA CGCCGCGAAC CTGGGCGTAT CGACCGGAGC AGTAGGCCAG GGCTTCGGCG	3078
CCGACTTCCA GCAGCGTCTG GTTCCACGCC TGGATCACGA CGCCCTCGCC CTCCTGGAGC	3138

-70-

GCACACAGCA ATTCCTGCTC ACCAACAACCT TGCTGCAGGA ACCCGTCGCC CTCGATCAGT	3198
GGGCGGCTCC GGAATTTCTG AACAAACAGCC TCAATCGCCA CCGATAGGAA CATCCGC	3255
ATG ACA CTG TCA CCT GAA AAG CAG CAC GTT CGA CCA CGC GAC GCC GCC Met Thr Leu Ser Pro Glu Lys Gln His Val Arg Pro Arg Asp Ala Ala 1 5 10 15	3303
GAC AAC GAT CCC GTC GCG GTT GCC CGT GGG CTA GCC GAA AAG TGG CGA Asp Asn Asp Pro Val Ala Val Ala Arg Gly Leu Ala Glu Lys Trp Arg 20 25 30	3351
GCC ACC GCC GTC GAG CGT GAT CGC GCC GGG GGT TCG GCA ACA GCC GAG Ala Thr Ala Val Glu Arg Asp Arg Ala Gly Gly Ser Ala Thr Ala Glu 35 40 45	3399
CGC GAA GAC CTG CGC GCG AGC GCG CTG CTG TCG CTC CTC GTC CCG CGC Arg Glu Asp Leu Arg Ala Ser Ala Leu Leu Ser Leu Leu Val Pro Arg 50 55 60	3447
GAA TAC GGC GGC TGG GGC GCA GAC TGG CCC ACC GCC ATC GAG GTC GTC Glu Tyr Gly Gly Trp Gly Ala Asp Trp Pro Thr Ala Ile Glu Val Val 65 70 75 80	3495
CGC GAA ATC GCG GCA GCC GAT GGA TCT TTG GGA CAC CTG TTC GGA TAC Arg Glu Ile Ala Ala Ala Asp Gly Ser Leu Gly His Leu Phe Gly Tyr 85 90 95	3543
CAC CTC ACC AAC GCC CCG ATG ATC GAA CTG ATC GGC TCG CAG GAA CAA His Leu Thr Asn Ala Pro Met Ile Glu Leu Ile Gly Ser Gln Glu Gln 100 105 110	3591
GAA GAA CAC CTG TAC ACC CAG ATC GCG CAG AAC AAC TGG TGG ACC GGA Glu Glu His Leu Tyr Thr Gln Ile Ala Gln Asn Asn Trp Trp Thr Gly 115 120 125	3639
AAT GCC TCC AGC GAG AAC AAC AGC CAC GTG CTG GAC TGG AAG GTC AGC Asn Ala Ser Ser Glu Asn Asn Ser His Val Leu Asp Trp Lys Val Ser 130 135 140	3687
GCC ACC CCG ACC GAA GAC GGC GGC TAC GTG CTC AAT GGC ACG AAG CAC Ala Thr Pro Thr Glu Asp Gly Gly Tyr Val Leu Asn Gly Thr Lys His 145 150 155 160	3735
TTC TGC AGC GGC GCC AAG GGG TCG GAC CTG CTG TTC GTG TTC GGC GTC Phe Cys Ser Gly Ala Lys Gly Ser Asp Leu Leu Phe Val Phe Gly Val 165 170 175	3783
GTC CAG GAT GAT TCT CCG CAG CAG GGT GCG ATC ATT GCT GCC GCT ATC Val Gln Asp Asp Ser Pro Gln Gln Gly Ala Ile Ile Ala Ala Ala Ile 180 185 190	3831
CCG ACA TCG CGG GCT GGC GTT ACG CCC AAC GAC GAC TGG GCC GCC ATC Pro Thr Ser Arg Ala Gly Val Thr Pro Asn Asp Asp Trp Ala Ala Ile 195 200 205	3879
GGC ATG CGG CAG ACC GAC AGC GGT TCC ACG GAC TTC CAC AAC GTC AAG Gly Met Arg Gln Thr Asp Ser Gly Ser Thr Asp Phe His Asn Val Lys 210 215 220	3927
GTC GAG CCT GAC GAA GTG CTG GGC GCG CCC AAC GCC TTC GTT CTC GCC Val Glu Pro Asp Glu Val Leu Gly Ala Pro Asn Ala Phe Val Leu Ala	3975



-71-

225	230	235	240	
TTC ATA CAA TCC GAG CGC GGC AGC CTC	TTC GCG CCC ATA GCG CAA TTG	4023		
Phe Ile Gln Ser Glu Arg Gly Ser Leu	Phe Ala Pro Ile Ala Gln Leu			
245	250	255		
ATC TTC GCC AAC GTC TAT CTG GGG ATC GCG CAC GGC GCA CTC GAT GCC	4071			
Ile Phe Ala Asn Val Tyr Leu Gly Ile Ala His Gly Ala Leu Asp Ala				
260	265	270		
GCC AGG GAG TAC ACC CGT ACC CAG GCG AGG CCC TGG ACA CCG GCC GGT	4119			
Ala Arg Glu Tyr Thr Arg Thr Gln Ala Arg Pro Trp Thr Pro Ala Gly				
275	280	285		
ATT CAA CAG GCA ACC GAG GAT CCC TAC ACC ATC CGC TCC TAC GGT GAG	4167			
Ile Gln Gln Ala Thr Glu Asp Pro Tyr Thr Ile Arg Ser Tyr Gly Glu				
290	295	300		
TTC ACC ATC GCA TTG CAG GGA GCT GAC GCC GCC GCC CGT GAA GCG GCC	4215			
Phe Thr Ile Ala Leu Gln Gly Ala Asp Ala Ala Ala Arg Glu Ala Ala				
305	310	315	320	
CAC CTG CTG CAG ACG GTG TGG GAC AAG GGC GAC GCG CTC ACC CCC GAG	4263			
His Leu Leu Gln Thr Val Trp Asp Lys Gly Asp Ala Leu Thr Pro Glu				
325	330	335		
GAC CGC GGC GAA CTG ATG GTG AAG GTC TCG GGA GTC AAA GCG TTG GCC	4311			
Asp Arg Gly Glu Leu Met Val Lys Val Ser Gly Val Lys Ala Leu Ala				
340	345	350		
ACC AAC GCC GCC CTC AAC ATC AGC AGC GGC GTC TTC GAG GTG ATC GGC	4359			
Thr Asn Ala Ala Leu Asn Ile Ser Ser Gly Val Phe Glu Val Ile Gly				
355	360	365		
GCG CGC GGA ACA CAT CCC AGG TAC GGT TTC GAC CGC TTC TGG CGC AAC	4407			
Ala Arg Gly Thr His Pro Arg Tyr Gly Phe Asp Arg Phe Trp Arg Asn				
370	375	380		
GTG CGC ACC CAC TCC CTG CAC GAC CCG GTG TCC TAC AAG ATC GCC GAC	4455			
Val Arg Thr His Ser Leu His Asp Pro Val Ser Tyr Lys Ile Ala Asp				
385	390	395	400	
GTC GGC AAG CAC ACC TTG AAC GGT CAA TAC CCG ATT CCC GGC TTC ACC	4503			
Val Gly Lys His Thr Leu Asn Gly Gln Tyr Pro Ile Pro Gly Phe Thr				
405	410	415		
TCC TGAGGATCTG AGGCGCTGAT CGAGGCCGAG GCCACCGCGC GGCCGAGTCG	4556			
Ser				
CGAATCGCCC GCCGATACTC AGCTTCTCCA TACGTACGGG TGCACACAAG GAGATATTGT	4616			
CAAGACCTGT GGATGAGGGT GTTTCAGGCG ACCTCCGTTT CGCTTGATTG GTCGGGCTCA	4676			
GCGGGTGAGA TGTCGATGGG TCGTTCGAGC AGCTTGCCCTT TGTGGAACAC CGCGCCGGCA	4736			
CGGACCAGCG CGACCAGATG GGGGGCGTTG ACCGCCGCCA GCGGGCTTGT GCGGCGTCGA	4796			
TCAGCTTGTA GGCCATGGCA ATCCCGCTGC GACGTGACCC AGGGCCCTTG GTGACCTTGG	4856			
TTCGCAACCG CACGGTCGCA AACGTCGATT CGATCGGATT CGTAGTGCGC AAGTGGATCC	4916			

-72-

```

AGTGCTCGGC CGGGTACCGG TAGAACTCCA GGAGCACGTC GCGTCGTCG ACGATCTTGG 4976
CGACCGCCTT GGGGTACTTC GCGCCGTAAT CTACCTCGAA GGCCTTGATC GCGACCTGGG 5036
CCTTGTCGAT GTCCTCGGCG TTGTAGATTT CCCGCATCGC CGCGGTCGCA CCTGGATGAG 5096
CCGACTTGGG CAGCGCAGCA AGCACATTGG CCTGCTTGTG AAACCAGCAG CGCTGTTCAC 5156
GGGTATCCGG AAACACCTCC CGCAGTGCCT TCCAGAACCC CAGCGCCCCA TCACCGACGG 5216
CCAGCACCGG GCGGGTCATC CCGCGGCGTC GGCATGAGCG CAGCAGATCA GCCCAGGACT 5276
CTGTGGACTC CCGGAACCCA TCGGTGAGCG CGACGAGCTC CTTGCGGCCG TCGGCGCGGA 5336
CGCCGATCAT CACGAGCAAG CACAGCTTCT CCTGCTCCAG GCGGACATTG AGATGGATGC 5396
CGTCGACCCA TAGGTACACG AAATCGGTGC CCGAGAGATC CCGGTCGGCG AAGGCCTTCG 5456
CCTCGTCCTG CCATTGCGCG GTCAGCCGGG TGATCGTCGA GGCCGACAGC CCGGCACCAG 5516
TGCCGAGGAA CTGCTCCAA 5535

```

## (2) INFORMATION FOR SEQ ID NO:2:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 454 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```

Met Thr Gln Gln Arg Gln Met His Leu Ala Gly Phe Phe Ser Ala Gly
 1           5           10           15
Asn Val Thr His Ala His Gly Ala Trp Arg His Thr Asp Ala Ser Asn
          20           25           30
Asp Phe Leu Ser Gly Lys Tyr Tyr Gln His Ile Ala Arg Thr Leu Glu
          35           40           45
Arg Gly Lys Phe Asp Leu Leu Phe Leu Pro Asp Gly Leu Ala Val Glu
          50           55           60
Asp Ser Tyr Gly Asp Asn Leu Asp Thr Gly Val Gly Leu Gly Gly Gln
          65           70           75           80
Gly Ala Val Ala Leu Glu Pro Ala Ser Val Val Ala Thr Met Ala Ala
          85           90           95
Val Thr Glu His Leu Gly Leu Gly Ala Thr Ile Ser Ala Thr Tyr Tyr
          100          105          110
Pro Pro Tyr His Val Ala Arg Val Phe Ala Thr Leu Asp Gln Leu Ser
          115          120          125
Gly Gly Arg Val Ser Trp Asn Val Val Thr S r Leu Asn Asp Ala Glu
          130          135          140
Ala Arg Asn Phe Gly Ile Asn Gln His Leu Glu His Asp Ala Arg Tyr

```

-73-

145	150	155	160
Asp Arg Ala Asp Glu Phe Leu Glu Ala Val Lys Lys Leu Trp Asn Ser	165	170	175
Trp Asp Glu Asp Ala Leu Val Leu Asp Lys Ala Ala Gly Val Phe Ala	180	185	190
Asp Pro Ala Lys Val His Tyr Val Asp His His Gly Glu Trp Leu Asn	195	200	205
Val Arg Gly Pro Leu Gln Val Pro Arg Ser Pro Gln Gly Glu Pro Val	210	215	220
Ile Leu Gln Ala Gly Leu Ser Pro Arg Gly Arg Arg Phe Ala Gly Lys	225	230	235
Trp Ala Glu Ala Val Phe Ser Leu Ala Pro Asn Leu Glu Val Met Gln	245	250	255
Ala Thr Tyr Gln Gly Ile Lys Ala Glu Val Asp Ala Ala Gly Arg Asp	260	265	270
Pro Asp Gln Thr Lys Ile Phe Thr Ala Val Met Pro Val Leu Gly Glu	275	280	285
Ser Gln Ala Val Ala Gln Glu Arg Leu Glu Tyr Leu Asn Ser Leu Val	290	295	300
His Pro Glu Val Gly Leu Ser Thr Leu Ser Ser His Thr Gly Ile Asn	305	310	315
Leu Ala Ala Tyr Pro Leu Asp Thr Pro Ile Lys Asp Ile Leu Arg Asp	325	330	335
Leu Gln Asp Arg Asn Val Pro Thr Gln Leu His Met Phe Ala Ala Ala	340	345	350
Thr His Ser Glu Glu Leu Thr Leu Ala Glu Met Gly Arg Arg Tyr Gly	355	360	365
Thr Asn Val Gly Phe Val Pro Gln Trp Ala Gly Thr Gly Glu Gln Ile	370	375	380
Ala Asp Glu Leu Ile Arg His Phe Glu Gly Gly Ala Ala Asp Gly Phe	385	390	395
Ile Ile Ser Pro Ala Phe Leu Pro Gly Ser Tyr Asp Glu Phe Val Asp	405	410	415
Gln Val Val Pro Val Leu Gln Asp Arg Gly Tyr Phe Arg Thr Glu Tyr	420	425	430
Gln Gly Asn Thr Leu Arg Asp His Leu Gly Leu Arg Val Pro Gln Leu	435	440	445
Gln Gly Gln Pro Ser	450		

-74-

## (2) INFORMATION FOR SEQ ID NO:3:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 416 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

```

Met Thr Leu Ser Pro Glu Lys Gln His Val Arg Pro Arg Asp Ala Ala
 1           5           10           15
Asp Asn Asp Pro Val Ala Val Ala Arg Gly Leu Ala Glu Lys Trp Arg
      20           25           30
Ala Thr Ala Val Glu Arg Asp Arg Ala Gly Gly Ser Ala Thr Ala Glu
      35           40           45
Arg Glu Asp Leu Arg Ala Ser Ala Leu Leu Ser Leu Leu Val Pro Arg
      50           55           60
Glu Tyr Gly Gly Trp Gly Ala Asp Trp Pro Thr Ala Ile Glu Val Val
      65           70           75           80
Arg Glu Ile Ala Ala Ala Asp Gly Ser Leu Gly His Leu Phe Gly Tyr
      85           90           95
His Leu Thr Asn Ala Pro Met Ile Glu Leu Ile Gly Ser Gln Glu Gln
      100          105          110
Glu Glu His Leu Tyr Thr Gln Ile Ala Gln Asn Asn Trp Trp Thr Gly
      115          120          125
Asn Ala Ser Ser Glu Asn Asn Ser His Val Leu Asp Trp Lys Val Ser
      130          135          140
Ala Thr Pro Thr Glu Asp Gly Gly Tyr Val Leu Asn Gly Thr Lys His
      145          150          155          160
Phe Cys Ser Gly Ala Lys Gly Ser Asp Leu Leu Phe Val Phe Gly Val
      165          170          175
Val Gln Asp Asp Ser Pro Gln Gln Gly Ala Ile Ile Ala Ala Ala Ile
      180          185          190
Pro Thr Ser Arg Ala Gly Val Thr Pro Asn Asp Asp Trp Ala Ala Ile
      195          200          205
Gly Met Arg Gln Thr Asp Ser Gly Ser Thr Asp Phe His Asn Val Lys
      210          215          220
Val Glu Pro Asp Glu Val Leu Gly Ala Pro Asn Ala Phe Val Leu Ala
      225          230          235          240
Phe Ile Gln Ser Glu Arg Gly Ser Leu Phe Ala Pro Ile Ala Gln Leu
      245          250          255
Ile Phe Ala Asn Val Tyr Leu Gly Ile Ala His Gly Ala Leu Asp Ala
      260          265          270

```

-75-

Ala Arg Glu Tyr Thr Arg Thr Gln Ala Arg Pro Trp Thr Pro Ala Gly  
 275 280 285

Ile Gln Gln Ala Thr Glu Asp Pro Tyr Thr Ile Arg Ser Tyr Gly Glu  
 290 295 300

Phe Thr Ile Ala Leu Gln Gly Ala Asp Ala Ala Arg Glu Ala Ala  
 305 310 315 320

His Leu Leu Gln Thr Val Trp Asp Lys Gly Asp Ala Leu Thr Pro Glu  
 325 330 335

Asp Arg Gly Glu Leu Met Val Lys Val Ser Gly Val Lys Ala Leu Ala  
 340 345 350

Thr Asn Ala Ala Leu Asn Ile Ser Ser Gly Val Phe Glu Val Ile Gly  
 355 360 365

Ala Arg Gly Thr His Pro Arg Tyr Gly Phe Asp Arg Phe Trp Arg Asn  
 370 375 380

Val Arg Thr His Ser Leu His Asp Pro Val Ser Tyr Lys Ile Ala Asp  
 385 390 395 400

Val Gly Lys His Thr Leu Asn Gly Gln Tyr Pro Ile Pro Gly Phe Thr  
 405 410 415

Ser

## (2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 5535 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: double  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

- (ix) FEATURE:  
 (A) NAME/KEY: CDS  
 (B) LOCATION: 2148..3245

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GCATGCACGT CGCGCCGACG CATTGCGCG CACGGCTCCG GGCAGTTC TC GCGGCGCTGG	60
AGGCACGGAT GGGCACCCTC AACGA ACTCA CCCAA ACCAC GCCGATAGCG ATCCTCGCCG	120
AAACCCTCGG CTACAGCCCT CAGACATTGG AAGCTCATGC GCGACGCATC CGGATCGACC	180
TTTGACACGCT ACGTGGCGAC GCGGCTGGAC TGACGCTGGA GGTCCGACCC GACGTGTGTG	240
GTGTAGCGCC GCTTAACGGG TGC GCACGGC GGGACATCGG CCAGCTGGCT TGCCCCCTCT	300
CCGCAGGTAG TCGACCACCC CTTCCCGCAG CGGTCCGAGG TGATCGACCG TTAGGGTCAT	360
TTGCTCGCAG ATCGGCTGAT GTTGCCGATC GACGTGGTCG ACGGGACACG CTCGCGATTG	420

-76-

GCATGGCGTC CGGTGCATAC ACGACGATCT AACCAGATCG ACGGTTTTGA GCGTCGGTCA	480
ACGTCGACTC GATGCGCCGT GCGAGTGAGA TCCTTTGTGG TGCTTGGCTA TTGACCTCGA	540
CAAGGATAGA GATTGGAAGG ACCTCGGATC GACCCAAATG CGGACGGCCG GCAGCGGCGA	600
AGGCGGCCAA GTCATCGGCA CCGTCACCGT CACCTTGACC CGACGTGCCC CGTGGTTCAA	660
GGCCTGAATT TGGCTGGTGG AGCATTGAAA TCAGGTGAAG TTTAACGGTG GGCACACCCC	720
GGGGGTGGGG GTGAGACTGC TTAGCGACAG GAATCTAGCC ATGATTGACA TTAAAGGAC	780
GCATACGCGA TGA CTCAACA ACGACAAATG CATCTGGCCG GTTCTTCTC GGCCGGCAAT	840
GTGACTCATG CACATGGGGC GTGGCGGCAC ACGGACGCGT CGAATGACTT TCTGTCGGGG	900
AAGTACTACC AACACATCGC CCGTACTCTG GAGCGCGGCA AGTTCGATCT GTTGTCTCTG	960
CCTGACGGGT TGGCCGTCGA GGACAGCTAC GGGGACAACC TGGACACCGG TGTCGGCCTG	1020
GGCGGGCAGG GTGCAGTCGC CTTGGAGCCG GCCAGTGTGG TCGCAACCAT GGCCGCGGTG	1080
ACCGAGCACC TGGGTCTTGG GGCAACCATT TCGGCGACCT ACTATCCCC GTATCACGTT	1140
GCTCGGGTGT TCGCGACGCT CGATCAGTTG TCAGGGGGTC GGGTGTCTCTG GAACGTCGTC	1200
ACCTCGCTCA ACGACGCTGA AGCGCGCAAC TTCGGCATT AATCAGCATCT GGAACACGAC	1260
GGCCGCTATG ACCGCGCCGA TGAGTTCTTG GAAGCGGTCA AGAACTCTG GAACAGCTGG	1320
GACGAGGACG CCCTCGTGCT GGACAAGGCG GCCGGCGTGT TCGCCGATCC CGCGAAGGTG	1380
CACTACGTCG ATCACCACGG GGAGTGCGTG AATGTGCGCG GACCTCTGCA GGTACCGCGT	1440
TCACCTCAGG GTGAGCCGGT GATCCTGCAG GCCGCGCTGT CGCCCCGGGG TCGGCGCTTC	1500
GCCGGGAAGT GGGCCGAGGC CGTCTTCAGT CTTGCACCCA ACCTCGAGGT GATGCAGGCC	1560
ACCTACCAGG GCATCAAAGC CGAGGTCGAC GCTGCGGGG GCGATCCCGA TCAGACGAAA	1620
ATCTTCACCG CCGTGATGCC GGTACTCGGC GAAAGCCAGG CGGTGGCACA GGAACGACTG	1680
GAATATCTCA ACAGTCTGGT CCATCCGGAA GTGGGACTGT CGACGCTATC CAGTCACACC	1740
GGCATCAACC TGGCGGCGTA CCCTCTCGAC ACTCCGATCA AGGACATCCT GCGGGATCTG	1800
CAGGATCGGA ATGTCCCGAC GCAACTGCAC ATGTTCCGGC CCGCAACGCA CAGCGAAGAG	1860
CTCACGCTGG CGGAAATGGG TCGGCGCTAT GGAACCAACG TGGGGTTCGT TCCTCAGTGG	1920
GCCGGTACCG GGGAGCAGAT CGCTGACGAG CTGATCCGCC ACTTCGAGGG CGGCGCCGCG	1980
GATGGTTTCA TCATCTCTCC GGCCTTCCTG CCGGGCTCCT ACGACGAGTT CGTCGACCAG	2040
GTGGTTCCGG TTCTGCAGGA TCGCGGCTAC TTCCGCACCG AGTACCAGGG CAACACTCTG	2100
CGCGACCACT TGGGTCTGCG CGTACCACAA CTGCAAGGAC AACCTTC ATG ACA AGC	2156
	Met Thr Ser
	1
CGC GTC GAC CCC GCA AAC CCC GGT TCA GAA CTC GAT TCC GCC ATC CGC	2204
Arg Val Asp Pro Ala Asn Pro Gly S r Glu Leu Asp Ser Ala Ile Arg	

-77-

5	10	15	
GAC ACA CTG ACC TAC AGC AAC TGC CCG GTA CCC AAC GCT CTG CTC ACG Asp Thr Leu Thr Tyr Ser Asn Cys Pro Val Pro Asn Ala Leu Leu Thr 20 25 30 35			2252
GCA TCG GAA TCG GGC TTC CTC GAC GCC GCC GGC ATC GAA CTC GAC GTC Ala Ser Glu Ser Gly Phe Leu Asp Ala Ala Gly Ile Glu Leu Asp Val 40 45 50			2300
CTC AGC GGC CAG CAG GGC ACG GTT CAT TTC ACC TAC GAC CAG CCT GCC Leu Ser Gly Gln Gln Gly Thr Val His Phe Thr Tyr Asp Gln Pro Ala 55 60 65			2348
TAC ACC CGT TTT GGG GGT GAG ATC CCG CCA CTG CTC AGC GAG GGG TTG Tyr Thr Arg Phe Gly Gly Glu Ile Pro Pro Leu Leu Ser Glu Gly Leu 70 75 80			2396
CGG GCA CCT GGG CGC ACG CGT CTA CTC GGC ATC ACC CCG CTC TTG GGG Arg Ala Pro Gly Arg Thr Arg Leu Leu Gly Ile Thr Pro Leu Leu Gly 85 90 95			2444
CGC CAG GGC TTC TTT GTC CGC GAC GAC AGC CCG ATC ACA GCG GCC GCC Arg Gln Gly Phe Phe Val Arg Asp Asp Ser Pro Ile Thr Ala Ala Ala 100 105 110 115			2492
GAC CTT GCC GGA CGT CGA ATC GGC GTC TCG GCC TCG GCA ATT CGC ATC Asp Leu Ala Gly Arg Arg Ile Gly Val Ser Ala Ser Ala Ile Arg Ile 120 125 130			2540
CTG CGC GGC CAG CTG GGC GAC TAC CTC GAG TTG GAT CCC TGG CGG CAA Leu Arg Gly Gln Leu Gly Asp Tyr Leu Glu Leu Asp Pro Trp Arg Gln 135 140 145			2588
ACG CTG GTA GCG CTG GGC TCG TGG GAG GCG CGC GCC TTG TTG CAC ACC Thr Leu Val Ala Leu Gly Ser Trp Glu Ala Arg Ala Leu Leu His Thr 150 155 160			2636
CTT GAG CAC GGT GAA CTG GGT GTG GAC GAC GTC GAG CTG GTG CCG ATC Leu Glu His Gly Glu Leu Gly Val Asp Asp Val Glu Leu Val Pro Ile 165 170 175			2684
AGC AGT CCT GGT GTC GAT GTT CCC GCT GAG CAG CTC GAA GAA TCG GCG Ser Ser Pro Gly Val Asp Val Pro Ala Glu Gln Leu Glu Glu Ser Ala 180 185 190 195			2732
ACC GTC AAG GGT GCG GAC CTC TTT CCC GAT GTC GCC CGC GGT CAG GCC Thr Val Lys Gly Ala Asp Leu Phe Pro Asp Val Ala Arg Gly Gln Ala 200 205 210			2780
GCG GTG TTG GCC AGC GGA GAC GTT GAC GCC CTG TAC AGT TGG CTG CCC Ala Val Leu Ala Ser Gly Asp Val Asp Ala Leu Tyr Ser Trp Leu Pro 215 220 225			2828
TGG GCC GGG GAG TTG CAA GCC ACC GGG GCC CGC CCA GTG GTG GAT CTC Trp Ala Gly Glu Leu Gln Ala Thr Gly Ala Arg Pro Val Val Asp Leu 230 235 240			2876
GGC CTC GAT GAG CGC AAT GCC TAC GCC AGT GTG TGG ACG GTC AGC AGC Gly Leu Asp Glu Arg Asn Ala Tyr Ala Ser Val Trp Thr Val Ser Ser 245 250 255			2924

-78-

GGG CTG GTT CGC CAG CGA CCT GGC CTT GTT CAA CGA CTG GTC GAC GCG Gly Leu Val Arg Gln Arg Pro Gly Leu Val Gln Arg Leu Val Asp Ala 260 265 270 275	2972
GCC GTC GAC GCC GGG CTG TGG GCA CGC GAT CAT TCC GAC GCG GTG ACC Ala Val Asp Ala Gly Leu Trp Ala Arg Asp His Ser Asp Ala Val Thr 280 285 290	3020
AGC CTG CAC GCC GCG AAC CTG GGC GTA TCG ACC GGA GCA GTA GGC CAG Ser Leu His Ala Ala Asn Leu Gly Val Ser Thr Gly Ala Val Gly Gln 295 300 305	3068
GGC TTC GGC GCC GAC TTC CAG CAG CGT CTG GTT CCA CGC CTG GAT CAC Gly Phe Gly Ala Asp Phe Gln Gln Arg Leu Val Pro Arg Leu Asp His 310 315 320	3116
GAC GCC CTC GCC CTC CTG GAG CGC ACA CAG CAA TTC CTG CTC ACC AAC Asp Ala Leu Ala Leu Leu Glu Arg Thr Gln Gln Phe Leu Leu Thr Asn 325 330 335	3164
AAC TTG CTG CAG GAA CCC GTC GCC CTC GAT CAG TGG GCG GCT CCG GAA Asn Leu Leu Gln Glu Pro Val Ala Leu Asp Gln Trp Ala Ala Pro Glu 340 345 350 355	3212
TTT CTG AAC AAC AGC CTC AAT CGC CAC CGA TAGGAACATC CGCATGACAC Phe Leu Asn Asn Ser Leu Asn Arg His Arg 360 365	3262
TGTCACCTGA AAAGCAGCAC GTTCGACCAC GCGACGCCGC CGACAAOGAT CCCGTCGCGG	3322
TTGCCCCGTGG GCTAGCCGAA AAGTGGCGAG CCACCGCCGT CGAGCGTGAT CGCGCCGGGG	3382
GTTCGGCAAC AGCCGAGCGC GAAGACCTGC GCGCGAGCGC GCTGCTGTCTG CTCCTCGTCC	3442
CGCGCGAATA CGGCGGCTGG GCGCGAGACT GGCCCAACGC CATCGAGGTC GTCCGCGAAA	3502
TCGCGGCAGC CGATGGATCT TTGGGACACC TGTTCCGATA CCACCTCACC AACGCCCCGA	3562
TGATCGAACT GATCGGCTCG CAGGAACAAG AAGAACACCT GTACACCCAG ATCGCGCAGA	3622
ACAACTGGTG GACCGGAAAT GCCTCCAGCG AGAACAACAG CCACGTGCTG GACTGGAAGG	3682
TCAGCGCCAC CCCGACCGAA GACGGCGGCT ACGTGCTCAA TGGCACGAAG CACTTCTGCA	3742
GCGGCGCCAA GGGGTCGGAC CTGCTGTTTC TGTTCCGGCT CGTCCAGGAT GATTCTCCGC	3802
AGCAGGGTGC GATCATTGCT GCCGCTATCC CGACATCGCG GGCTGGCGTT ACGCCCAACG	3862
ACGACTGGGC CGCCATCGGC ATGCGGCAGA CCGACAGCGG TTCCACGGAC TTCCACAACG	3922
TCAAGGTCCA GCCTGACGAA GTGCTGGGCG CGCCCAACGC CTTCGTCTCT GCCTTCATAC	3982
AATCCGAGCG CGGCAGCCTC TTCGCGCCCA TAGCGCAATT GATCTTCGCC AACGTCTATC	4042
TGGGGATCGC GCACGGCGCA CTCGATGCCG CCAGGGAGTA CACCCGTACC CAGGCGAGGC	4102
CCTGGACACC GGCCGGTATT CAACAGGCAA CCGAGGATCC CTACACCATC CGCTCCTACG	4162
GTGAGTTCAC CATCGCATTG CAGGGAGCTG ACGCCGCCGC CCGTGAAGCG GCCCACCTGC	4222
TGCAGACGGT GTGGGACAAG GCGGACGCGC TCACCCCCGA GGACCGCGGC GAACTGATGG	4282



-79-

TGAAGGTCTC	GGGAGTCAAA	GCGTTGGCCA	CCAACGCCGC	CCTCAACATC	AGCAGCGGCG	4342
TCTTCGAGGT	GATCGGCGCG	CGCGGAACAC	ATCCCAGGTA	CGGTTTCGAC	CGCTTCTGGC	4402
GCAACGTGCG	CACCCACTCC	CTGCACGACC	CGGTGTCTTA	CAAGATCGCC	GACGTCGGCA	4462
AGCACACCTT	GAACGGTCAA	TACCCGATTC	CCGGCTTCAC	CTCCTGAGGA	TCTGAGGCGC	4522
TGATCGAGGC	CGAGGCCACC	GCGCGGCCGA	GTCGCGAATC	GCCCGCCGAT	ACTCAGCTTC	4582
TCCATACGTA	CGGGTGCACA	CAAGGAGATA	TTGTCAAGAC	CTGTGGATGA	GGGTGTTTCA	4642
GGCGACCTCC	GTTTCGCTTG	ATTCTGCGGG	CTCAGCGGGT	GAGATGTCGA	TGGGTCGTTC	4702
GAGCAGCTTG	CCTTTGTGGA	ACACCGCGCC	GGCAGCGACC	AGCGCGACCA	GATGGGGGGC	4762
GTTGACCGCC	GCCAGCGGGC	TTGTGCGGCG	TCGATCAGCT	TGTAGGCCAT	GGCAATCCCG	4822
CTGCGACGTG	ACCCAGGGCC	CTTGGTGACC	TTGGTTCGCA	ACCGCACGGT	CGCAAACGTC	4882
GATTCGATCG	GATTCGTAGT	GCGCAAGTGG	ATCCAGTGCT	CGGCCGGGTA	CCGGTAGAAC	4942
TCCAGGAGCA	CGTCGGCGTC	GTCGACGATC	TTGGCGACCG	CCTTGGGGTA	CTTCGCGCCG	5002
TAATCTACCT	CGAAGGCCTT	GATCGCGACC	TGGGCCTTGT	CGATGTCCTC	GGCGTTGTAG	5062
ATTTCCCGCA	TCGCCGCGGT	CGCACCTGGA	TGAGCCGACT	TGGGCAGCGC	AGCAAGCACA	5122
TTGGCCTGCT	TGTGAAACCA	GCAGCGCTGT	TCACGGGTAT	CCGGAACAC	CTCCCGCAGT	5182
GCCTTCCAGA	ACCCAGCGC	CCCATCACCG	ACGCCAGCA	CCGGGGCGGT	CATCCCGCGG	5242
CGTCGGCATG	AGCGCAGCAG	ATCAGCCCAC	GACTCTGTGG	ACTCCCGGAA	CCCATCGGTG	5302
AGCGCGACGA	GCTCCTTGCG	GCCGTCGGCG	CGGACGCGA	TCATCACGAG	CAAGCACAGC	5362
TTCTCCTGCT	CCAGGCGGAC	ATTGAGATGG	ATGCCGTCGA	CCCATAGGTA	CACGAAATCG	5422
GTGCCCCAGA	GATCCCGGTC	GGCGAAGGCC	TTGCCTCGT	CCTGCCATTG	CGCGGTCAGC	5482
CGGGTGATCG	TCGAGGCCGA	CAGCCCGGCA	CCAGTGCCGA	GGAAGTGCTC	CAA	5535

## (2) INFORMATION FOR SEQ ID NO:5:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 365 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Met	Thr	Ser	Arg	Val	Asp	Pro	Ala	Asn	Pro	Gly	Ser	Glu	Leu	Asp	Ser
1				5				10						15	
Ala	Ile	Arg	Asp	Thr	Leu	Thr	Tyr	Ser	Asn	Cys	Pro	Val	Pro	Asn	Ala
			20					25						30	
Leu	L	u	Thr	Ala	Ser	Glu	S	r	Gly	Phe	Leu	Asp	Ala	Ala	Gly
			35						40					45	

-80-

Leu Asp Val Leu Ser Gly Gln Gln Gly Thr Val His Phe Thr Tyr Asp  
 50 55 60  
 Gln Pro Ala Tyr Thr Arg Phe Gly Gly Glu Ile Pro Pro Leu Leu Ser  
 65 70 75 80  
 Glu Gly Leu Arg Ala Pro Gly Arg Thr Arg Leu Leu Gly Ile Thr Pro  
 85 90 95  
 Leu Leu Gly Arg Gln Gly Phe Phe Val Arg Asp Asp Ser Pro Ile Thr  
 100 105 110  
 Ala Ala Ala Asp Leu Ala Gly Arg Arg Ile Gly Val Ser Ala Ser Ala  
 115 120 125  
 Ile Arg Ile Leu Arg Gly Gln Leu Gly Asp Tyr Leu Glu Leu Asp Pro  
 130 135 140  
 Trp Arg Gln Thr Leu Val Ala Leu Gly Ser Trp Glu Ala Arg Ala Leu  
 145 150 155 160  
 Leu His Thr Leu Glu His Gly Glu Leu Gly Val Asp Asp Val Glu Leu  
 165 170 175  
 Val Pro Ile Ser Ser Pro Gly Val Asp Val Pro Ala Glu Gln Leu Glu  
 180 185 190  
 Glu Ser Ala Thr Val Lys Gly Ala Asp Leu Phe Pro Asp Val Ala Arg  
 195 200 205  
 Gly Gln Ala Ala Val Leu Ala Ser Gly Asp Val Asp Ala Leu Tyr Ser  
 210 215 220  
 Trp Leu Pro Trp Ala Gly Glu Leu Gln Ala Thr Gly Ala Arg Pro Val  
 225 230 235 240  
 Val Asp Leu Gly Leu Asp Glu Arg Asn Ala Tyr Ala Ser Val Trp Thr  
 245 250 255  
 Val Ser Ser Gly Leu Val Arg Gln Arg Pro Gly Leu Val Gln Arg Leu  
 260 265 270  
 Val Asp Ala Ala Val Asp Ala Gly Leu Trp Ala Arg Asp His Ser Asp  
 275 280 285  
 Ala Val Thr Ser Leu His Ala Ala Asn Leu Gly Val Ser Thr Gly Ala  
 290 295 300  
 Val Gly Gln Gly Phe Gly Ala Asp Phe Gln Gln Arg Leu Val Pro Arg  
 305 310 315 320  
 Leu Asp His Asp Ala Leu Ala Leu Leu Glu Arg Thr Gln Gln Phe Leu  
 325 330 335  
 Leu Thr Asn Asn Leu Leu Gln Glu Pro Val Ala Leu Asp Gln Trp Ala  
 340 345 350  
 Ala Pro Glu Phe Leu Asn Asn Ser Leu Asn Arg His Arg  
 355 360 365

-81-

CLAIMS

1. A recombinant DNA molecule containing a gene which encodes a biocatalyst capable of desulfurizing a fossil fuel which contains organic sulfur molecules.
- 5 2. The recombinant DNA molecule of Claim 1 wherein said fossil fuel is petroleum.
3. A recombinant DNA molecule containing a gene of a strain of Rhodococcus rhodochrous bacteria wherein said gene encodes a biocatalyst capable of desulfur-  
10 izing a fossil fuel which contains organic sulfur molecules.
4. The recombinant DNA molecule of Claim 3 wherein the gene is derived from the IGTS8 strain of Rhodococcus rhodochrous bacteria.
- 15 5. The recombinant DNA molecule of Claim 3 wherein said fossil fuel is petroleum.
6. Purified DNA derived from a strain of Rhodococcus rhodochrous bacteria wherein said DNA encodes a biocatalyst capable of desulfurizing a fossil fuel  
20 which contains organic sulfur molecules.
7. Purified DNA of Claim 6 wherein said DNA is derived from the IGTS8 strain of Rhodococcus rhodochrous bacteria.

-82-

8. Purified DNA derived from a strain of Rhodococcus rhodochrous bacteria wherein said DNA encodes a biocatalyst capable of desulfurizing petroleum which contains organic sulfur molecules.
- 5 9. Purified DNA of Claim 8 wherein said DNA is derived from the IGTS8 strain of Rhodococcus rhodochrous bacteria.
- 10 10. A recombinant DNA vector containing a DNA molecule containing a gene which encodes a biocatalyst capable of desulfurizing a fossil fuel which contains organic sulfur molecules.
- 15 11. A recombinant DNA vector containing a DNA molecule containing the gene of a strain of Rhodococcus rhodochrous bacteria wherein said gene encodes a biocatalyst capable of desulfurizing a fossil fuel which contains organic sulfur molecules.
12. The recombinant DNA vector of Claim 11 wherein the gene is derived from the IGTS8 strain of Rhodococcus rhodochrous bacteria.
- 20 13. A DNA plasmid vector containing a DNA derived from a microorganism wherein said DNA encodes a biocatalyst capable of desulfurizing a fossil fuel which contains organic sulfur molecules.

-83-

14. A DNA plasmid vector containing a DNA derived from a strain of Rhodococcus rhodochrous bacteria wherein said DNA encodes a biocatalyst capable of desulfurizing a fossil fuel which contains organic sulfur molecules.
15. The DNA plasmid vector of Claim 14 wherein the DNA is derived from the IGTS8 strain of Rhodococcus rhodochrous bacteria.
16. A DNA plasmid containing a recombinant DNA molecule containing the gene of a microorganism wherein said gene encodes a biocatalyst capable of desulfurizing a fossil fuel which contains organic sulfur molecules.
17. A DNA plasmid containing a recombinant DNA molecule containing the gene of a strain of Rhodococcus rhodochrous bacteria wherein said gene encodes a biocatalyst capable of desulfurizing a fossil fuel which contains organic sulfur molecules.
18. The DNA plasmid of Claim 17 wherein said gene is derived from the IGTS8 strain of Rhodococcus rhodochrous bacteria.
19. A DNA plasmid containing an isolated DNA derived from a strain of Rhodococcus rhodochrous bacteria wherein said DNA encodes a biocatalyst capable of desulfurizing a fossil fuel which contains organic sulfur molecules.

-84-

20. The DNA plasmid of Claim 19 wherein the isolated DNA is derived from the IGTS8 strain of Rhodococcus rhodochrous bacteria.
21. Plasmid pTOXI-1.
- 5 22. Plasmid pTOXI-2.
23. A microorganism containing a recombinant DNA plasmid containing a gene which expresses a biocatalyst capable of desulfurizing a fossil fuel which contains organic sulfur molecules.
- 10 24. A microorganism containing a recombinant DNA plasmid containing the gene of a strain of Rhodococcus rhodochrous bacteria wherein said microorganism expresses a biocatalyst capable of desulfurizing a fossil fuel which contains organic sulfur molecules.
- 15 25. The microorganism of Claim 24 wherein the gene is derived from the IGTS8 strain of Rhodococcus rhodochrous bacteria.
- 20 26. A microorganism containing a DNA derived from a strain of Rhodococcus rhodochrous bacteria wherein said transformed microorganism expresses a biocatalyst capable of desulfurizing a fossil fuel which contains organic sulfur molecules.
- 25 27. The microorganism of Claim 26 wherein the DNA plasmid is derived from the IGTS8 strain of Rhodococcus rhodochrous bacteria.

-85-

28. Complemented host organism CPE-648 containing plasmid pTOXI-1.
29. Complemented host organism CPE-648 containing plasmid pTOXI-2.
- 5 30. A method of desulfurizing a fossil fuel which contains organic sulfur molecules through the use of an organism containing a recombinant DNA plasmid containing a gene which expresses a biocatalyst capable of desulfurizing a fossil fuel which contains organic sulfur molecules, comprising the steps of:
- 10 a) contacting the fossil fuel with the microorganism; and
- b) incubating the fossil fuel and microorganism mixture under conditions sufficient to bring about the catalytic cleavage of organic carbon-sulfur bonds,
- 15 whereby the organic sulfur content of the fossil fuel is significantly reduced.
31. A method of desulfurizing a fossil fuel which contains organic sulfur molecules through the use of a microorganism containing a recombinant DNA plasmid containing the gene of a strain of Rhodococcus rhodochrous bacteria wherein said microorganism expresses a biocatalyst capable of desulfurizing a
- 20 fossil fuel which contains organic sulfur molecules, comprising the steps of:
- 25 a) contacting the fossil fuel with the microorganism; and

-86-

- b) incubating the fossil fuel and micro-  
organism mixture under conditions suf-  
ficient to bring about the catalytic clea-  
vage of organic carbon-sulfur bonds,  
5 whereby the organic sulfur content of the fossil  
fuel is significantly reduced.
32. The transformed microorganism of Claim 31 wherein  
the gene is derived from the IGTS8 strain of Rhodo-  
coccus rhodochrous bacteria.
- 10 33. A method of desulfurizing a fossil fuel which con-  
tains organic sulfur molecules through the use of a  
microorganism containing a DNA plasmid containing  
DNA derived from a strain of Rhodococcus rhodochrous  
bacteria wherein said microorganism expresses a  
15 biocatalyst capable of desulfurizing a fossil fuel  
which contains organic sulfur molecules, comprising  
the steps of:
- a) contacting the fossil fuel with said mi-  
croorganism; and
- 20 b) incubating the fossil fuel and transformed  
microorganism mixture under conditions  
sufficient to bring about the catalytic  
cleavage of organic carbon-sulfur bonds,  
whereby the organic sulfur content of the fossil  
25 fuel is significantly reduced.
34. The method of desulfurizing a fossil fuel of Claim  
33 wherein the fossil fuel is petroleum.



-87-

35. The method of desulfurizing a fossil fuel of Claim 33 wherein the DNA is derived from the IGTS8 strain of Rhodococcus rhodochrous bacteria.
- 5 36. A nucleic acid probe capable of hybridizing to all or a portion of a recombinant DNA molecule containing the gene of a biocatalyst capable of desulfurizing a fossil fuel which contains organic sulfur molecules.
- 10 37. A nucleic acid probe capable of hybridizing to all or a portion of a recombinant DNA molecule containing the gene of a strain of Rhodococcus rhodochrous bacteria wherein said gene encodes a biocatalyst capable of desulfurizing a fossil fuel which contains organic sulfur molecules.
- 15 38. A nucleic acid probe capable of hybridizing to all or a portion of a DNA derived from a strain of Rhodococcus rhodochrous bacteria wherein said DNA encodes a biocatalyst capable of desulfurizing a fossil fuel which contains organic sulfur molecules.
- 20 39. The nucleic acid sequence set forth in Sequence No. 1.
40. The nucleic acid sequence set forth in Sequence No. 2.
- 25 41. The nucleic acid sequence set forth in Sequence No. 3.

-88-

42. The nucleic acid sequence set forth in Sequence No. 4.
43. The nucleic acid sequence set forth in Sequence No. 5.
- 5 44. The DNA sequence for ORF-1.
45. The DNA sequence for ORF-2.
46. The DNA sequence for ORF-3.
47. The amino acid sequence for ORF-1.
48. The amino acid sequence for ORF-2.
- 10 49. The amino acid sequence for ORF-3.
50. The DNA sequences set forth starting on page 66 through page 80.

1/12

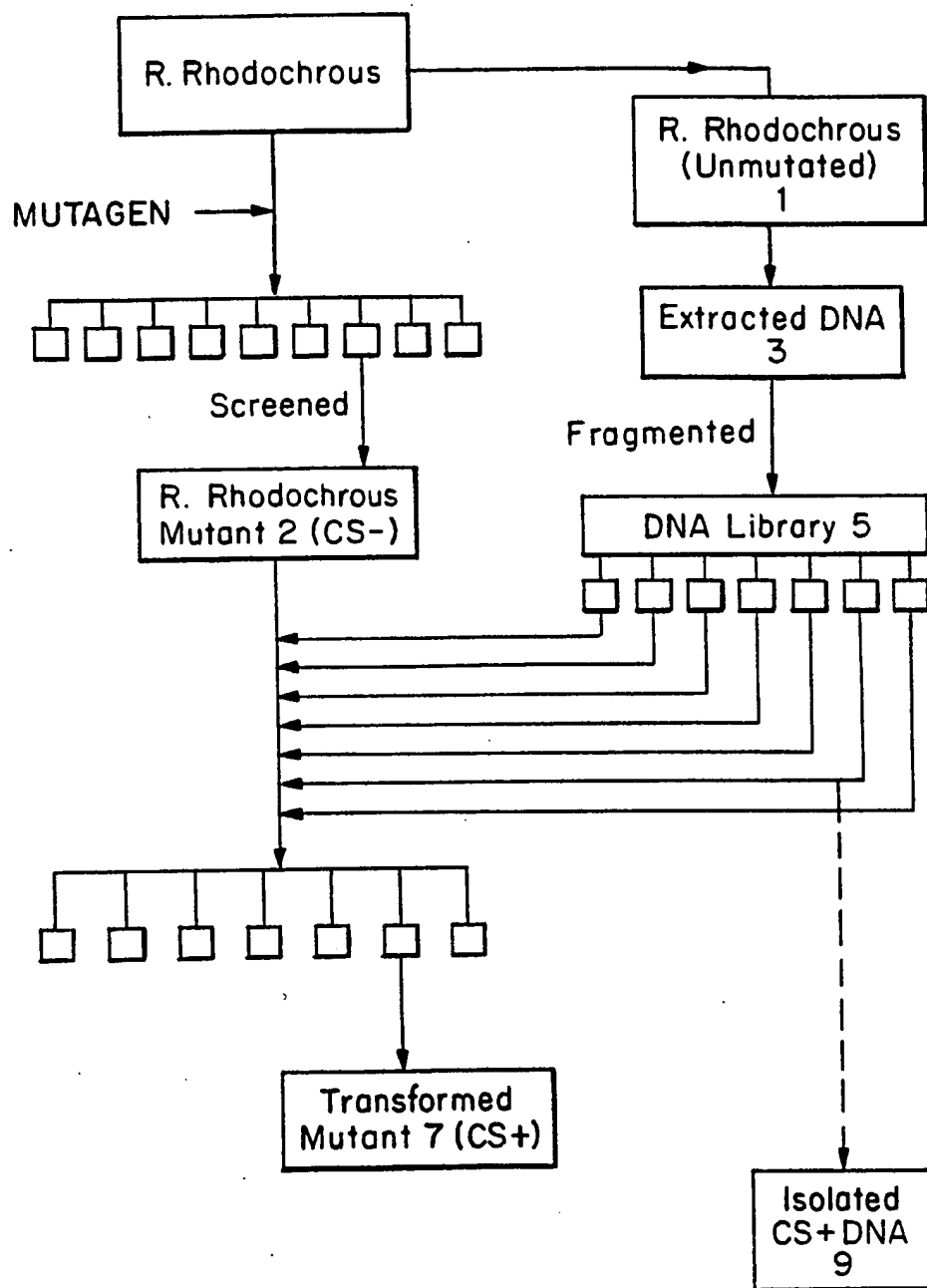


FIG. 1

2/12

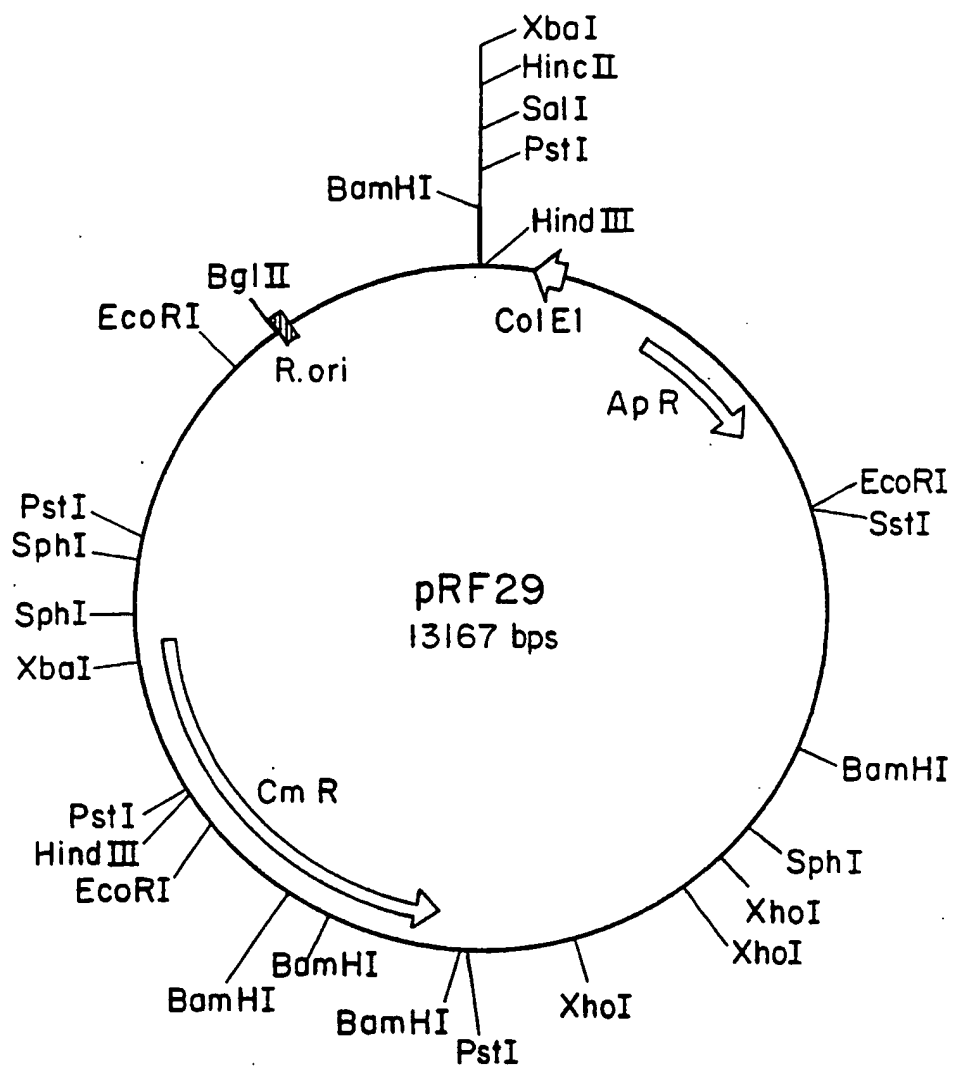


FIG. 2

3/12

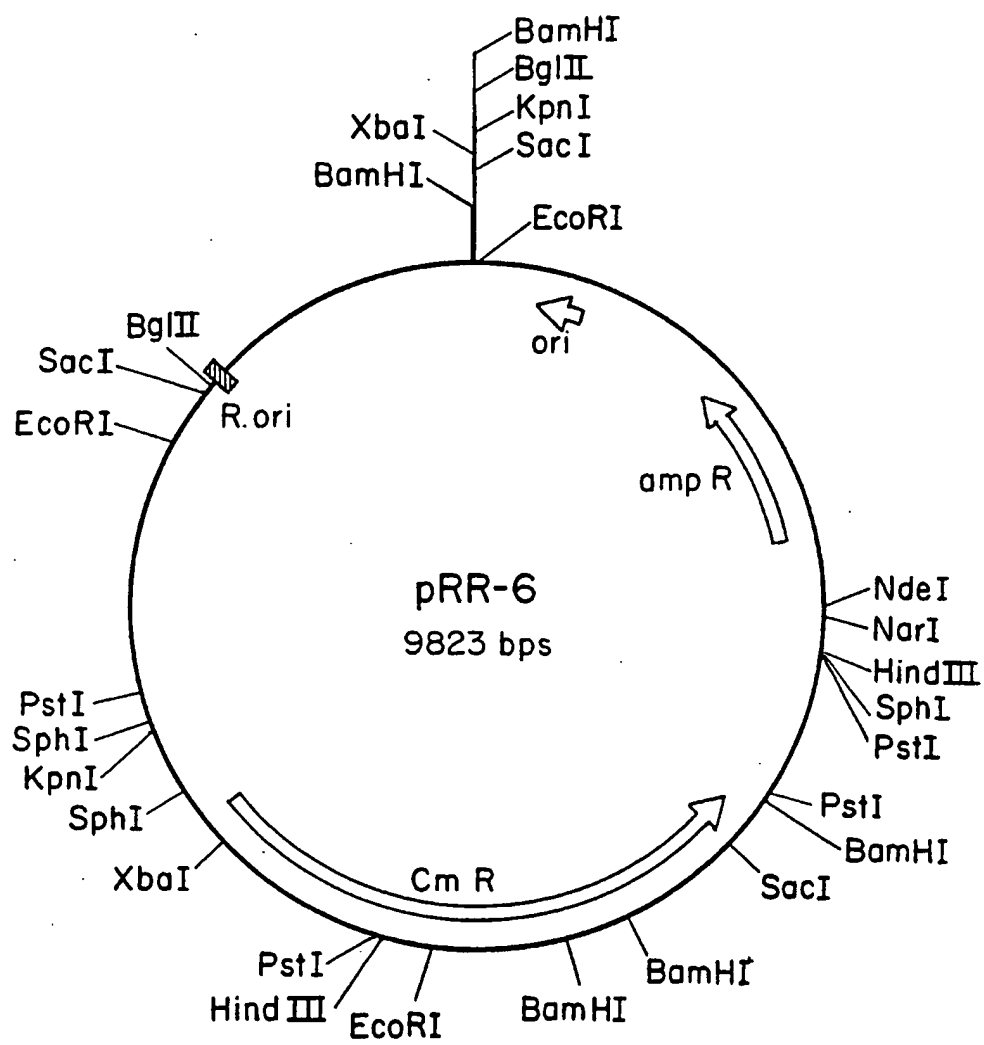


FIG. 3

4/12

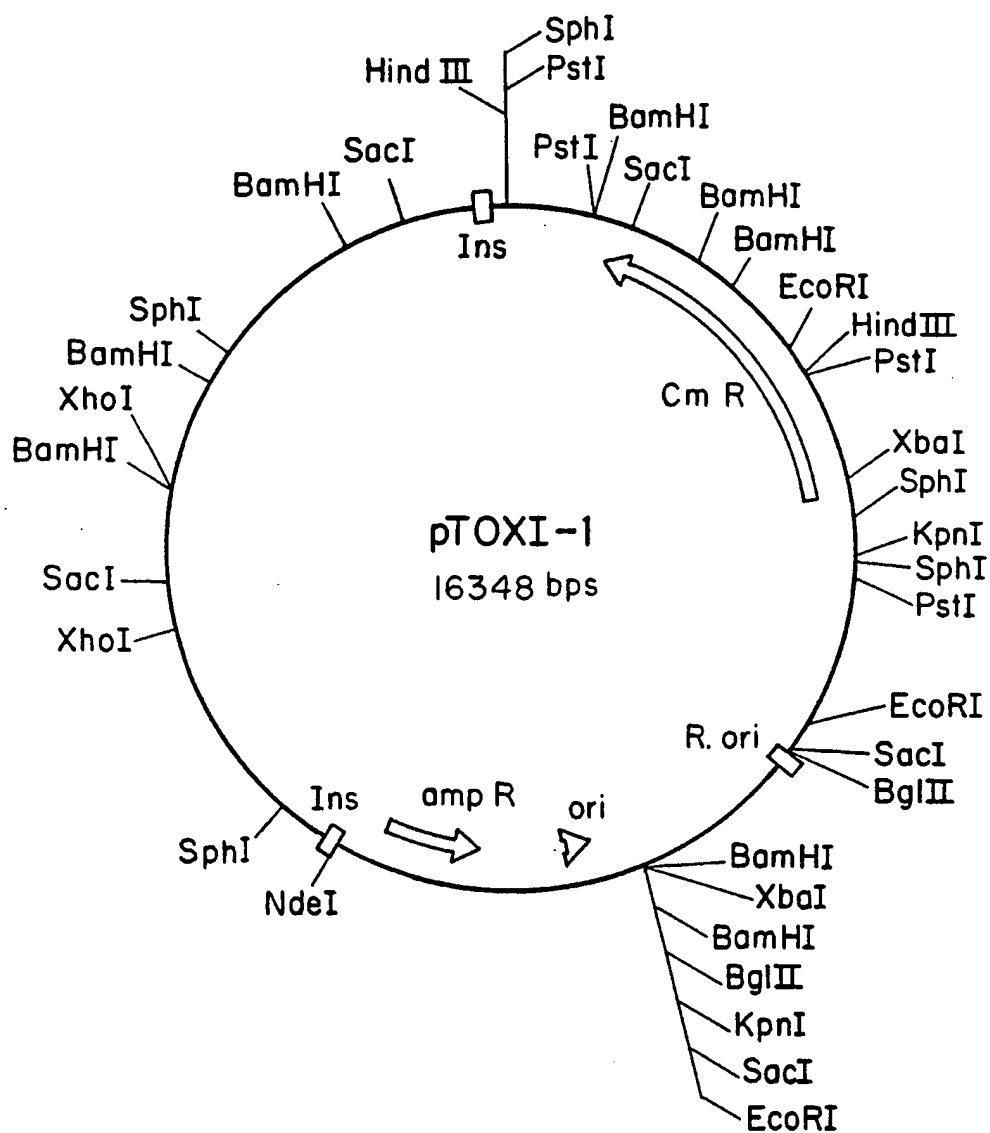
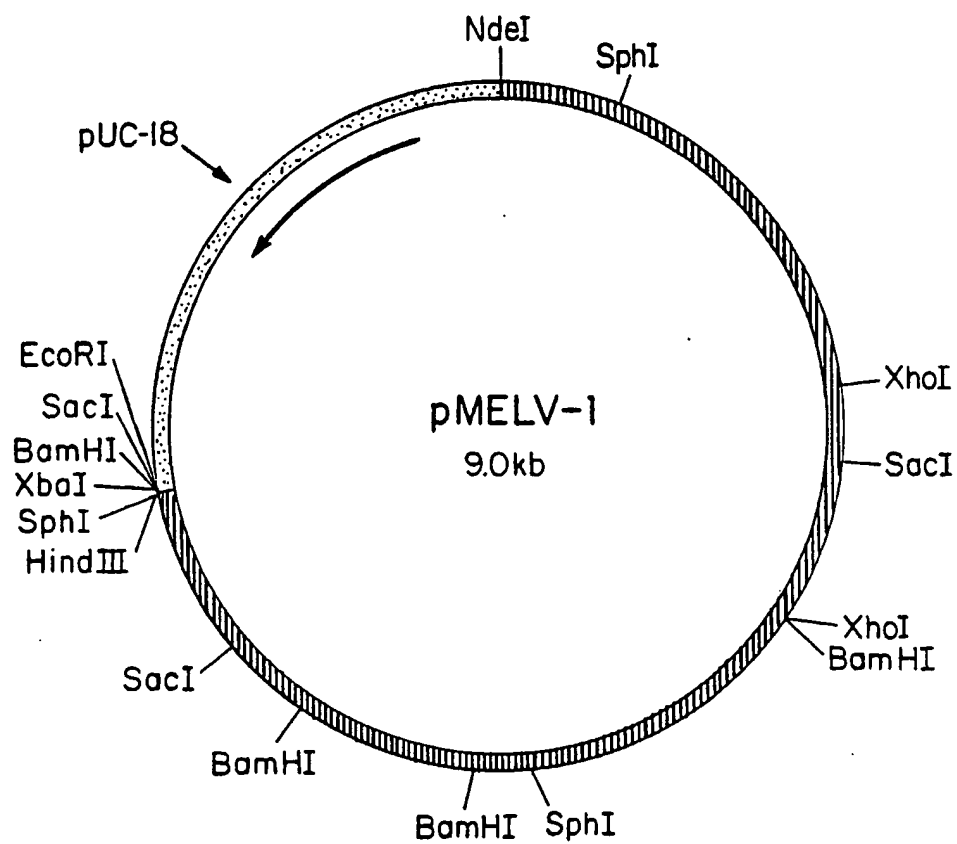


FIG. 4

5/12

*FIG. 5*

6/12

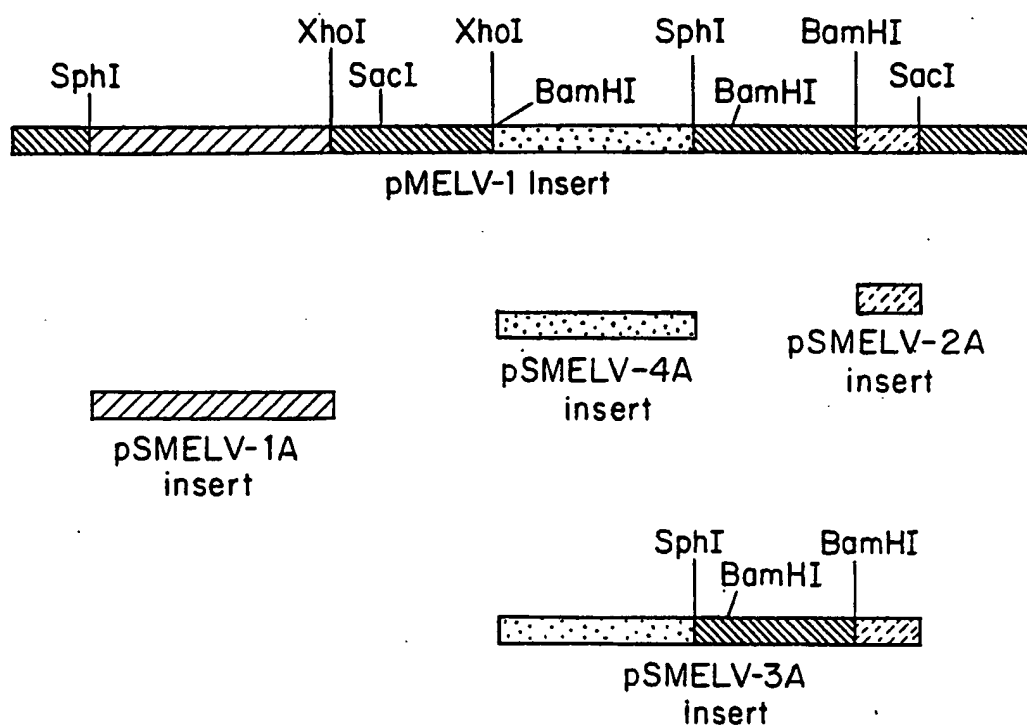


FIG. 6



7/12

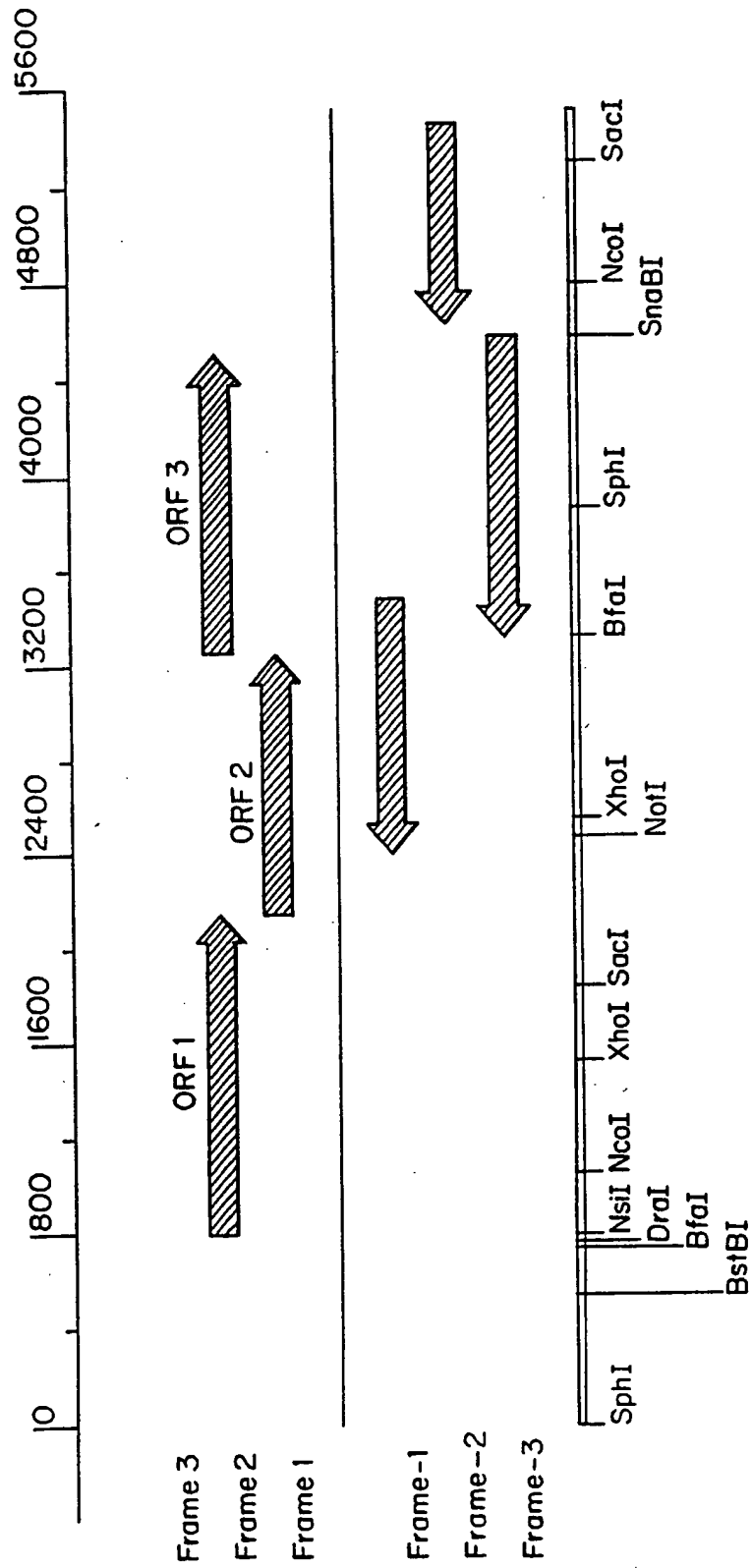


FIG. 7

8/12

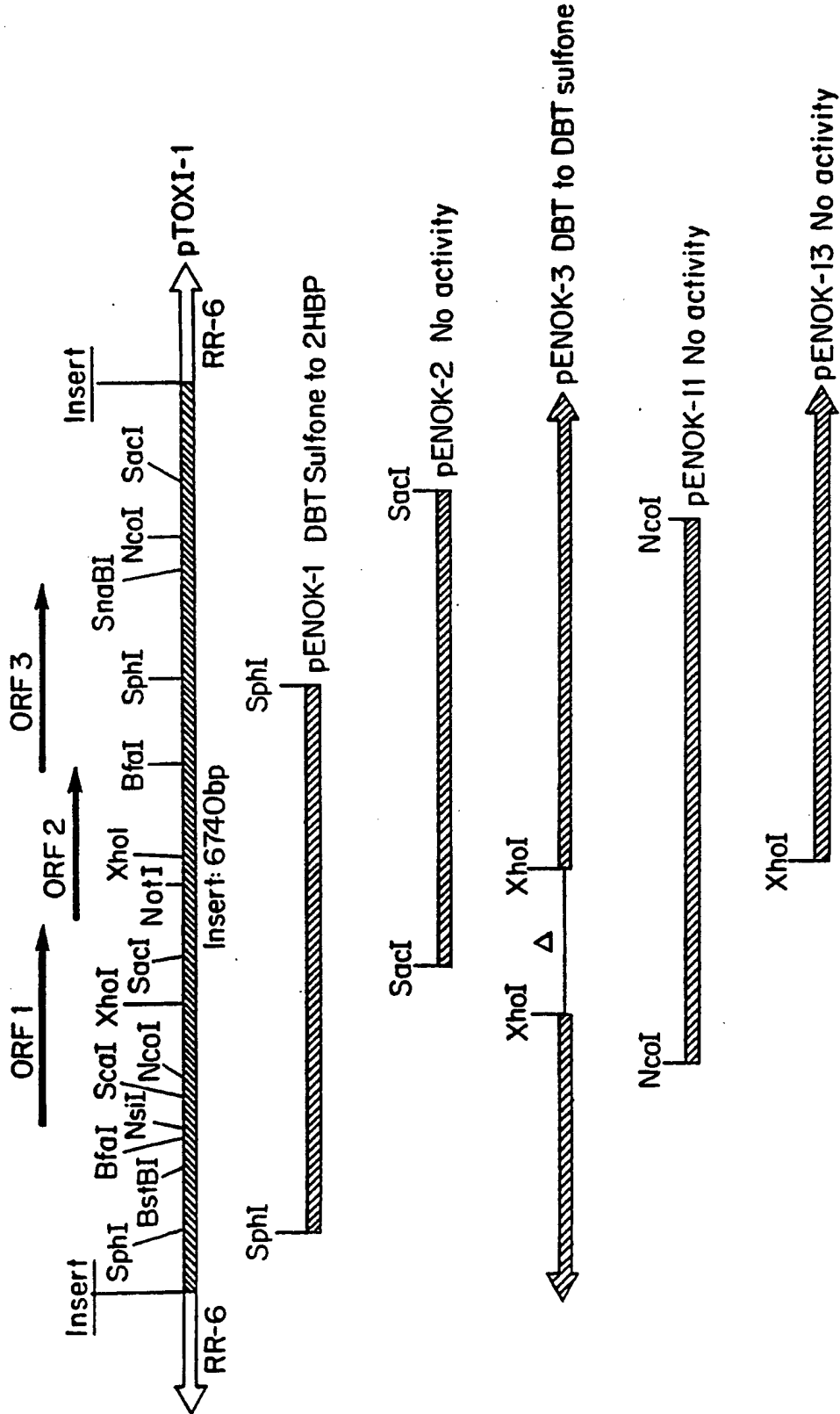


FIG. 8A

9/12

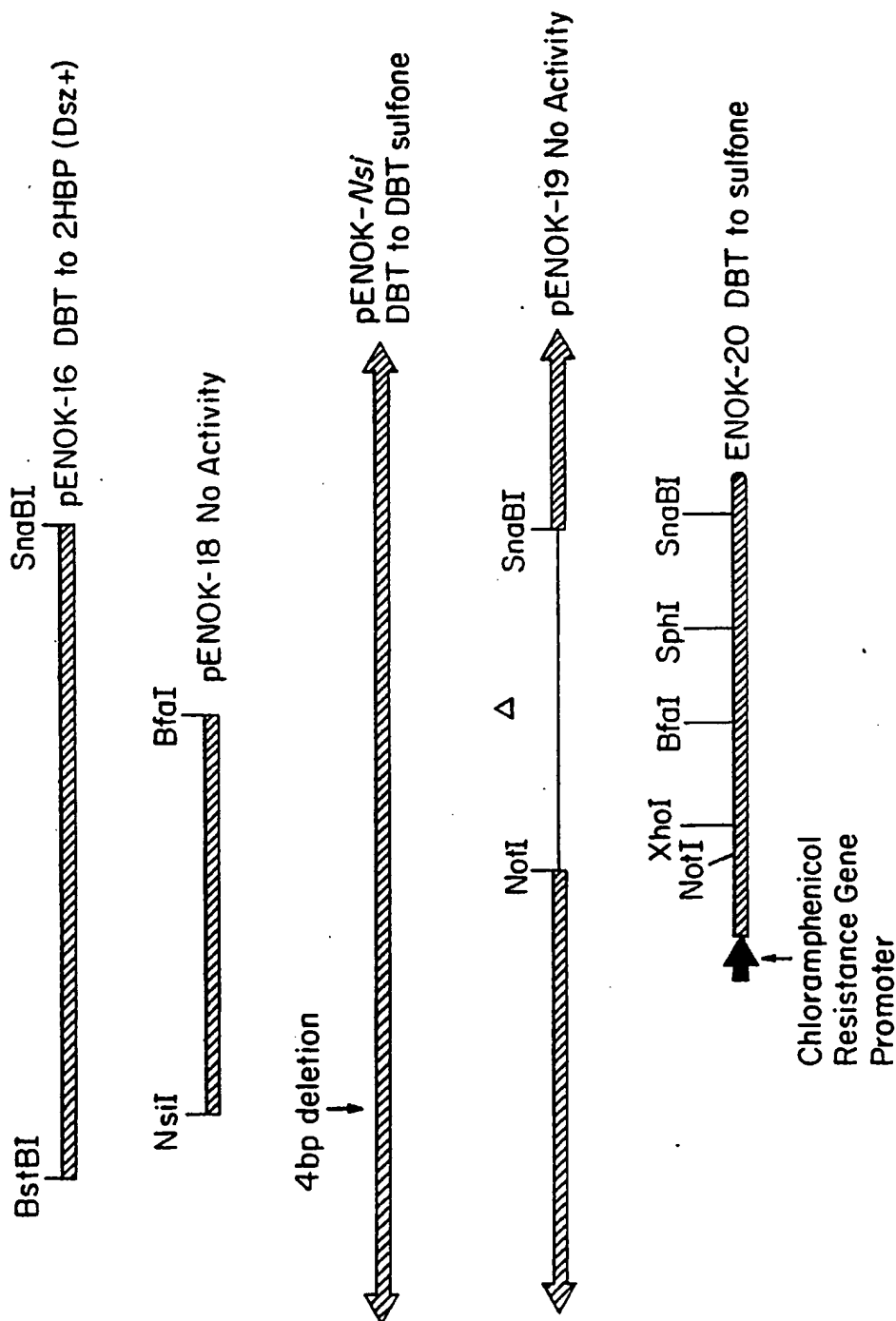


FIG. 8B

10/12

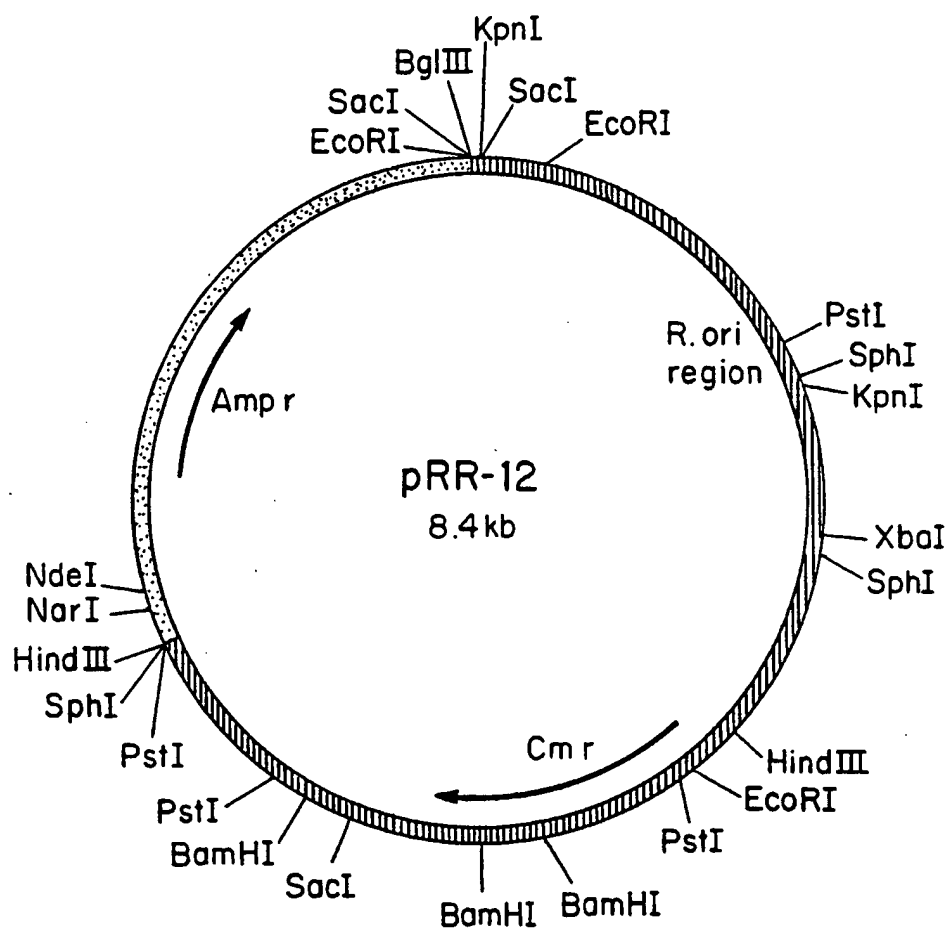
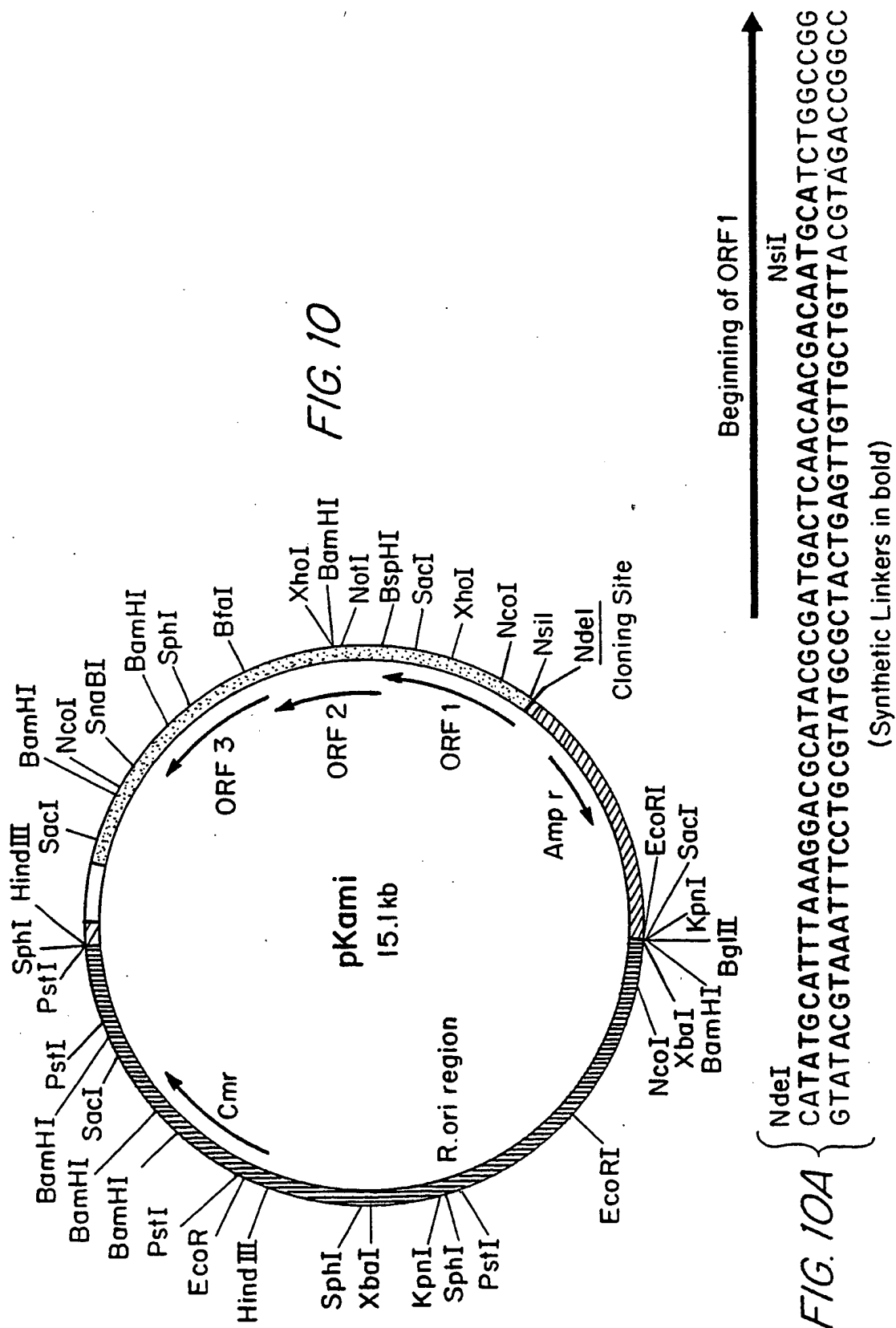


FIG. 9

11/12



12/12

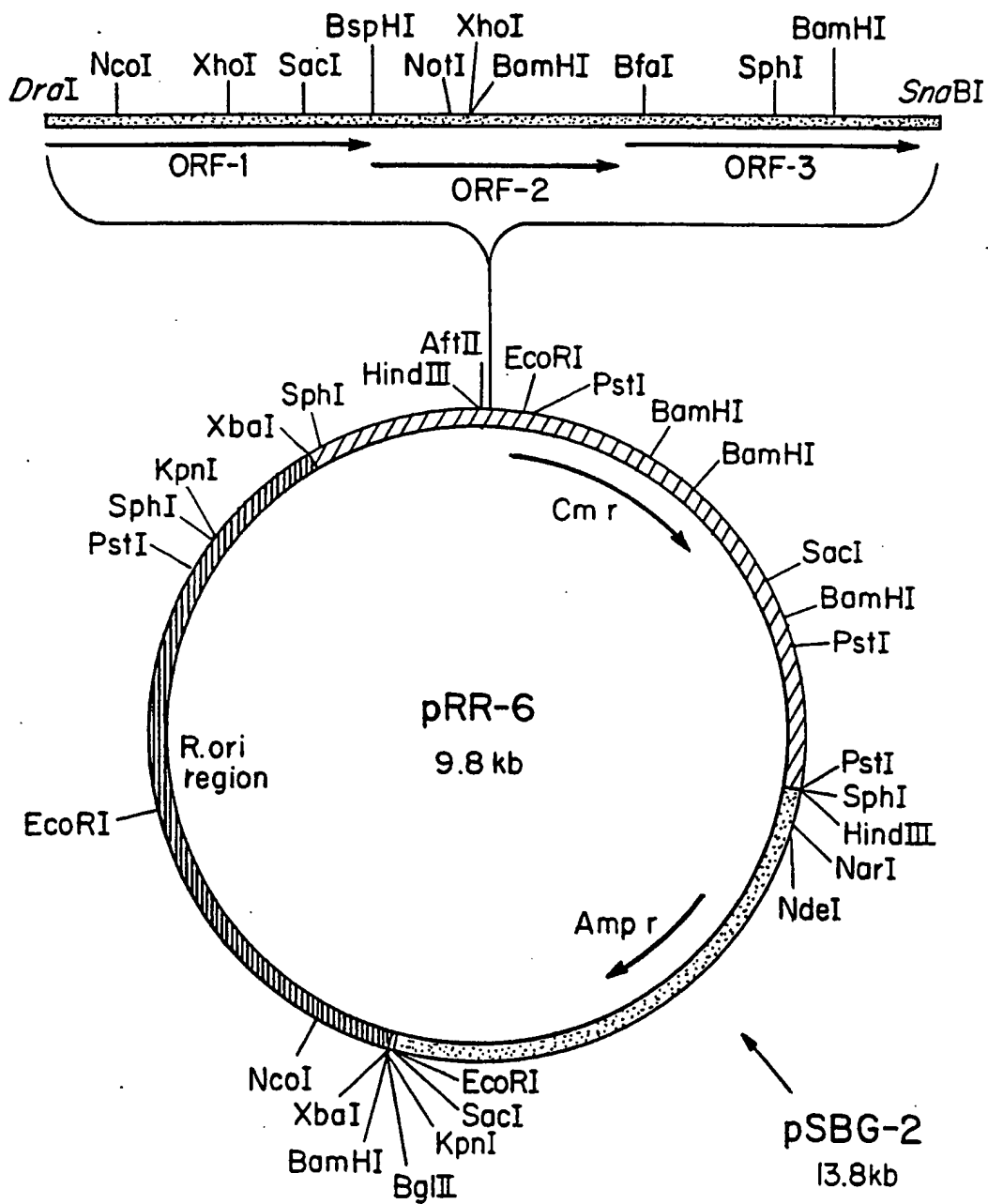


FIG. 11

## INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 93/06497

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 5 C12N15/52 C07K15/00 C10G32/00 C12S1/02 C12N1/21  
 //(C12N1/21,C12R1:01,C12R1:19)

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)  
 IPC 5 C12N C07K C10G C12R C12S

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	EP,A,0 445 896 (INSTITUTE OF GAS TECHNOLOGY) 11 September 1991 see page 12, line 17 - line 23 ---	30-35
Y	TRENDS IN BIOTECHNOLOGY vol. 7, no. 4, April 1989, ELSEVIER SCIENCE PUBLISHERS, LTD., CAMBRIDGE, UK; pages 97 - 101 J.J. KILBANE 'Desulfurization of coal: the microbial solution' cited in the application see page 100, middle column, line 5 - right column, line 9 ---	30-35
P,Y	WO,A,92 16602 (ENVIRONEMENTAL BIOSCIENCE CORPORATION) 1 October 1992 see page 20, line 22 - page 21, line 3 --- -/--	30-35

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

## \* Special categories of cited documents:

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

12 November 1993

Date of mailing of the international search report

07. 12. 93

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2  
 NL - 2280 HV Rijswijk  
 Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,  
 Fax (+31-70) 340-3016

Authorized officer

HORNIG, H

# INTERNATIONAL SEARCH REPORT

Int: mal Application No  
PCT/US 93/06497

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,Y	WO,A,92 19700 (ENERGY BIOSYSTEM CORPORATION) 12 November 1992 see page 18, line 4 - line 15 -----	30-35



# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 93/06497

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A-0445896	11-09-91	US-A- 5132219	21-07-92
WO-A-9216602	01-10-92	US-A- 5232854	03-08-93
		AU-A- 1643992	21-10-92
		CN-A- 1064880	30-09-92
WO-A-9219700	12-11-92	AU-A- 2233992	21-12-92
		CN-A- 1066285	18-11-92